AAMRL-TR-90-066

EVALUATION OF THE INITIATION/PROMOTION POTENTIAL OF CTFE TRIMER ACID

C. S. Godin
C. D. Flemming
J. M. Drerup
H. G. Wall

NSI TECHNOLOGY SERVICES CORPORATION
P. O. BOX 31009
DAYTON, OH 45431-0009

NOVEMBER 1990

FINAL REPORT FOR THE PERIOD MARCH 1989 TO JULY 1990

HARRY G. ARMSTRONG AEROSPACE MEDICAL RESEARCH LABORATORY
HUMAN SYSTEMS DIVISION
AIR FORCE SYSTEMS COMMAND
WRIGHT-PATTERSON AIR FORCE BASE, OHIO 45433-6573

NOTICES

When U S Government drawings, specifications, or other data are used for any purpose other than a definitely related Government procurement operation, the Government thereby incurs no responsibility nor any obligation whatsoever, and the fact that the Government may have formulated, furnished, or in any way supplied the said drawings, specifications, or other data, is not to be regarded by implication or otherwise, as in any manner licensing the holder or any other person or corporation, or conveying any rights or permission to manufacture, use, or sell any patented invention that may in any way be related thereto.

Please do not request copies of this report from the Harry G. Armstrong Aerospace Medical Research Laboratory. Additional copies may be purchased from:

National Technical Information Service
5285 Port Royal Road
Springfield, Virginia 22161

Federal Government agencies and their contractors registered with Defense Technical Information Center should direct requests for copies of this report to:

Defense Technical Information Center
Cameron Station
Alexandria, Virginia 22314

TECHNICAL REVIEW AND APPROVAL

AAMRL-TR-90-066

The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

JAMES N. McDOUGAL, Maj, USAF, BSC
Deputy Director, Toxic Hazards Division
Harry G. Armstrong Aerospace Medical Research Laboratory

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1. AGENCY USE ONLY (Leave Blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	NOVEMBER 1990	Final Report, March 1989 – July 1990

4. TITLE AND SUBTITLE
Evaluation of the Initiation/Promotion Potential of CTFE Trimer Acid

5. FUNDING NUMBERS
PE 62202F
PR 6302
TA 630201
WU 63020171
Accession No.

6. AUTHOR(S)
C.S. Godin, C.D. Fleming, J.M. Drerup, H.G. Wall

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
NSI Technology Services Corporation
P.O. Box 31009
Dayton, OH 45431-0009

8. PERFORMING ORGANIZATION REPORT NUMBER
AAMRL-TR-90-066

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
AAMRL, Toxic Hazards Division
HSD, AFSC
Wright-Patterson AFB, OH 45433-6573

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

CTFE trimer acid is a metabolite of the 6-carbon oligomer of Halocarbon 3.1 oil, a nonflammable hydraulic fluid composed of perhalogenated oligomers of varying chain length. Administration of CTFE trimer acid by oral gavage for 3 months resulted in a slight increase in the rate of peroxisomal β-oxidation, but not relative liver weight. An increase in peroxisomal β-oxidation has been correlated with the formation of hepatic tumors. Therefore, the present study was designed to evaluate CTFE trimer acid for both tumor initiation and promoting ability. Male Sprague-Dawley rats (4 weeks old) were partially hepatectomized Groups of animals received a single dose of CTFE trimer acid as the initiator, followed by chronic phenobarbital administration, a known tumor promotor, for either 3 or 9 months. Diethylnitrosamine, a known tumor initiator, was administered as a single dose to separate groups of animals that were administered different doses of CTFE trimer acid as a promotor for either 3 or 9 months. Quantitative sterological analysis was performed on foci from liver sections stained for a variety of histological and histochemical markers. CTFE trimer acid does not possess tumor initiation ability However, a significant increase in foci/cm^2 and foci/cm^3 in livers of treated animals over those of control after 9 months of treatment clearly indicates that CTFE trimer acid has promoting activity. Interestingly, no increase in either β-oxidation or relative liver weight was noted in animals that received CTFE trimer acid as a tumor promotor.

14. SUBJECT TERMS
Chlorotrifluoroethylene Trimer Acid
Initiation
Peroxisome Proliferator
Hepatocarcinogenesis
Perhalogenated Fatty Acid
Promotion

15. NUMBER OF PAGES
43

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UL

PREFACE

This is one of a series of technical reports describing results of the experimental laboratory programs conducted at the Toxic Hazards Research Unit, NSI Technology Services Corporation-Environmental Sciences. This document serves as a final report on the evaluation of the initiation/promotion potential of chlorotrifluoroethylene (CTFE) trimer acid. The research described in this report began in March 1989 and was completed in July 1990 under U.S. Air Force Contract No. F33615-85-C-0532. During the initiation and conduct of these studies Melvin E. Andersen, Ph.D., Lt Col Harvey Clewell, III, Lt Col Michael B. Ballinger, and Maj James N. McDougal served consecutively as the Contract Technical Monitor for the U.S. Air Force, Harry G. Armstrong Aerospace Medical Research Laboratory.

The animals used in this study were handled in accordance with the principles stated in the *Guide for the Care and Use of Laboratory Animals*, prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, Department of Health and Human Services, National Institute of Health Publication #86-23, 1985, and the Animal Welfare Act of 1966, as amended.

The opinions contained herein are those of the authors and are not to be construed as official or reflecting the views of the Department of the Air Force. The use of trade names in this report does not constitute an official endorsement or approval of the use of such commercial hardware or software. This report may not be cited for purposes of advertisement.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ATPase	Adenosine triphosphatase
B.P.	Boiling point
CoA	Coenzyme A
CTFE	Chlorotrifluoroethylene
DEN	Diethylnitrosamine
DNA	Deoxyribonucleic acid
Eq. wt.	Equivalent weight
g	Gram
GGTase	Gamma-glutamyltranspeptidase
G6Pase	Glucose-6-phosphatase
h	Hour
HE	Hematoxylin and eosin
ip	Intraperitoneal
kg	Kilogram
µm	Micrometer
mg	Milligram
mL	Milliliter
mm	Millimeter
m.w.	Molecular weight
M	Molar
N	Normal
NTP	National Toxicology Program
p	Probability
PAS	Periodic acid/Schiff
PB	Phenobarbital
PFDA	Perfluoro-*n*-decanoic acid
SEM	Standard error of the mean

SECTION 1

INTRODUCTION

Halocarbon 3.1 oil is a hydraulic fluid consisting of chlorotrifluoroethylene (CTFE) oligomers of varying carbon chain lengths that is being considered for use by the Department of Defense. The chronic administration of Halocarbon 3.1 oil for 90 days by inhalation resulted in hepatomegaly and an increased number of peroxisomes within hepatocytes (Kirkead et al., 1990). A study in which different formulations of Halocarbon 3.1 oil and six- and eight-carbon oligomers of CTFE were administered by oral gavage for 14 days resulted in hepatomegaly and an increase in the rate of cyanide-insensitive peroxisomal β-oxidation of palmitoyl coenzyme A (CoA) (DelRaso, unpublished findings).

Many compounds cause an increase in the number of hepatic peroxisomes and are structural analogs of the hypolipidemic agent, clofibrate (Lalwani et al., 1983). The proliferative response is not restricted to hypolipidemic agents, however, because numerous industrial chemicals such as phthalate ester plasticizers (Reddy et al., 1976; Moody and Reddy, 1978), agricultural chemicals such as phenoxy acid herbicides (Vainio et al., 1983; Kawashima et al., 1984), and even a high-fat diet (Ishii et al., 1980) can induce hepatic peroxisomal proliferation. Several peroxisome proliferators have been shown to inhibit mitochondrial fatty acid oxidation in rat liver (Bone et al., 1982; Horie and Suga, 1985; Elcombe and Mitchell, 1986; Draye and Vamecq, 1987; Foxworthy and Eacho, 1988; Eacho and Foxworthy, 1988), which has suggested that inhibition of mitochondrial β-oxidation may induce peroxisome proliferation as an adaptive response (Sharma et al., 1988).

Recently, perfluoro-*n*-decanoic acid (PFDA), a compound structurally and chemically unrelated to known peroxisome proliferators, was shown to result in hepatomegaly (Olson et al., 1982), peroxisomal proliferation (Van Rafelghem, 1985), and a 20- to 40-fold increase in fatty acyl-CoA oxidase activity, the rate-limiting enzyme in the fatty acid oxidase system (Harrison et al., 1988). These findings and the fact that mammals can oxidize *n*-alkanes to the corresponding fatty acids (McCarthy, 1964), have led to the hypothesis that CTFE oligomers can be metabolized to perhalogenated fatty acids similar to PFDA.

In rodents, the chronic administration of peroxisome proliferators, such as hypolipidemic agents, causes an increase in benign and malignant hepatic tumors (Reddy et al., 1980; National Toxicology Program, 1976, 1982; Hartig et al., 1982). The trend for hepatocarcinogenic potency in rodents has been correlated with peroxisome proliferative potency (Reddy et al., 1980; Elcombe, 1985). Peroxisome proliferators have not demonstrated mutagenic potential and they fail to bind to DNA or induce its repair (Warren et al., 1980; Gupta et al., 1985). However, several peroxisome

proliferators have been shown to act as tumor-promoting agents (Reddy and Rao, 1978; Schulte-Hermann et al., 1981; Mochizuki et al., 1982).

Because of the correlation between peroxisome proliferation and hepatocarcinogenesis the U.S. Air Force requested that the following study be designed to provide information on the ability of the CTFE trimer acid to act either as a tumor initiator or promoter. The design of this study is based upon that described by Parnell et al. (1986), which utilized the male Sprague-Dawley rat.

SECTION 2

MATERIALS

Animals

Male Sprague-Dawley rats (three weeks of age) were purchased from Charles River Laboratories (Kingston, NY). Upon receipt the animals were quarantined, quality control tested, and found to be in acceptable health. Prior to surgical procedures the animals were group-housed (four per cage) in plastic cages containing hardwood-chip bedding and given a commercial diet (Purina Formulab 5008) and water ad libitum. Following surgery, the animals were housed singly. Ambient temperatures were maintained at 21 to 25 °C and the light/dark cycle was set at 12-h intervals (light cycle starting at 0700 h).

Test Materials

Chlorotrifluoroethylene trimer acid was purchased by the Air Force from Technolube Products, Inc., Ultrasystems, Inc., Irvine, CA. All solutions of CTFE trimer acid were prepared in sterile saline as the sodium salt and the pH was adjusted to 7.4. Pertinent data are provided below.

ID# (Lot No.) 10-86-40 IR# 14086
B.P. 82-85C/10^3 mm Hg
Eq. wt. 357.1, m.w. 363.5

Diethylnitrosamine (DEN, purity >98%) was supplied by Sigma Chemical Company, St. Louis, MO. A 10 mg/mL solution of DEN in saline was prepared by adding 99 mL of sterile normal saline directly through a rubber septum into a sealed vial containing 1 g of DEN. Doses were removed with a sterile syringe through the septum. Pertinent physical characteristic are provided below.

Synonym	*N*-nitrosodiethylamine
CAS Reg. No.	55-18-5
Vapor Pressure (mm Hg)	20 °C 0.81
	40 °C 3.10
Specific Gravity (g/mL)	0.942

Phenobarbital (PB, purity 99%) was supplied by Sigma Chemical Company, St. Louis, MO. Solutions of PB (0.05% in the drinking water) were prepared by adding 4N sodium hydroxide to a mixture of PB and water until all PB dissolved. The pH of the solution was adjusted to 7.4 by the addition of 4N hydrochloric acid and water was added to yield the final volume. Pertinent physical characteristics are provided below.

CAS Reg. No.	50-06-6
Melting Point	174-178 °C
Solubility	Water soluble

SECTION 3

EXPERIMENTAL APPROACH

Initiation Assessment

A total of seven groups (A through G) consisting of eight animals per group, and one group representing the age-matched negative control (Group H), consisting of four animals, was used. Animals were subjected to a two-thirds partial hepatectomy procedure (Higgins and Anderson, 1931) using isoflurane anesthesia, except for Group F (Table 1), which was sham hepatectomized and Group H, which received no surgery. The sham procedure consisted of a laparotomy only. Mortality following surgery reduced some groups to a total of six animals. The surgical procedure was followed 24 h later by a single intraperitoneal (ip) dose of DEN (10 mg/kg) to Group A. Groups B through F were administered CTFE trimer acid (98 mg/kg) by ip injection. This dose was determined by a physiologically based pharmacokinetic model for CTFE (Vinegar, personal communication). This dose was the amount required to bring the concentration of CTFE trimer acid in the livers of test animals in the present study to the amount present in the livers of animals exposed to a 90-day inhalation study with CTFE oligomers at an inhalation exposure of 0.25 mg/L. This exposure concentration was the level at which no significant drop in body weight was noted during the period of exposure (Kinkead et al., 1990). At various times following DEN or CTFE trimer acid administration (14 days for DEN and 1, 10, 20, or 30 days for CTFE trimer acid), all groups were administered PB (0.05%) in the drinking water for the remainder of the study. Three months following the beginning of PB administration, three or four animals from each group, depending on the extent of mortality following surgery, were euthanatized by CO_2 asphyxiation. Animals from Group G were not examined because mortality had reduced the number of animals to four. The terminal whole animal and liver weights were obtained from each animal. The remainder of the animals in each group were euthanatized nine months following the beginning of PB administration and similarly treated.

Promotion Assessment

There were seven groups consisting of eight animals per group (M through S) and one group consisting of four animals representing the age-matched negative control (Group T) in this portion of the study. The experimental animals were subjected to a two-thirds partial hepatectomy with isoflurane anesthesia, except for those in Group Q, which were sham-hepatectomized and Group T, which received no treatment. Between 20 and 24 h following hepatectomy, all animals received DEN (10 mg/kg) via ip injection, except for Groups Q and R, which received saline by the same route. Two weeks after these injections, PB (0.05%) was administered to Group M, whereas CTFE trimer acid was given by ip injection to Groups N through Q at the levels and frequency shown in Table 2. Groups R

and S received saline injections by the same route. Three or four animals from each group were euthanatized three months after the beginning of either PB or CTFE trimer acid treatment. The terminal whole body and liver weights were obtained from each animal. The remainder of the animals in each group were euthanatized nine months following the beginning of PB or trimer acid administration and similarly treated.

TABLE 1. EXPERIMENTAL DESIGN OF THE INITIATION PHASE

Group[a]	A	B	C	D	E	F	G	H
PH	+	+	+	+	+	–	+	–
Initiator	DEN[b]	ACID[c]	ACID[c]	ACID[c]	ACID[c]	ACID[c]	–	–
# Days[d]	14	1	10	20	30	30	–	–
Promoter	PB[e]	PB[e]	PB[e]	PB[e]	PB[e]	PB[e]	PB[e]	–

[a] PH = Partial hepatectomy
DEN = Diethylnitrosamine
ACID = CTFE trimer acid
PB = Phenobarbital

[b] DEN single dose ip, 10mg/kg in saline

[c] Trimer acid single ip dose, 98 mg/kg

[d] Number of days refers to the length of time between injection of either DEN or trimer acid and the beginning of PB administration.

[e] PB in drinking water (0.05%)

TABLE 2. EXPERIMENTAL DESIGN OF THE PROMOTION PHASE

Group	M	N	O	P	Q	R	S	T
PH	+	+	+	+	–	+	+	–
Initiator	DEN[a]	DEN[a]	DEN[a]	DEN[a]	–	–	DEN[a]	–
Promoter	PB[b]	ACID[c]	ACID[d]	ACID[e]	ACID[c]	–	–	–

[a] DEN single ip dose, 10 mg/kg in saline

[b] PB in drinking water (0.05%)

[c] Trimer acid (initial dose = 98 mg/kg, maintenance dose = 12.25 mg/kg every two weeks)

[d] Trimer acid (initial dose = 9.8 mg/kg, maintenance dose = 1.23 mg/kg every two weeks)

[e] Trimer acid (initial dose = 0.98 mg/kg, maintenance dose = 0.12 mg/kg every two weeks)

Histological and Histochemical Studies

Immediately after death, the liver was excised, weighed, and the liver lobules resected. A cross-section from the right anterior lobule was removed from animals euthanatized after three months of treatment and placed in buffered neutral formalin. A cross-section from both the right anterior and posterior lobules was removed from animals euthanatized after nine months of treatment, and placed in buffered neutral formalin. Following fixation each piece of liver was embedded in paraffin, and six serial sections (5-μm thick) were prepared from three separate areas within each paraffin block and stained as follows. The first section from each area was stained with hematoxylin and eosin (HE). The second serial section from each area was stained for the presence of iron as described by

Hirota and Williams (1979). The third serial section was stained for the presence of glycogen using the periodic acid/Schiff reaction (PAS) described by Bedi and Horobin (1976).

A separate piece of liver from the right anterior and posterior lobules of all study animals was frozen and serial frozen sections (10-μm thick) were prepared from three separate areas within each piece of liver and stained as follows. The first serial section was stained for the presence of gamma-glutamyltranspeptidase (GGTase) activity using the method described by Rutenburg et al. (1969). The second serial section was stained for the presence of adenosine triphosphatase (ATPase) activity according to the method described by Wachstein and Meisel (1957). The third serial section from each of the three areas was stained for the presence of glucose-6-phosphatase (G6Pase) activity by the method described by Wachstein and Meisel (1958).

Image Analysis

All stained slides were examined for the presence of foci. The liver section area and the foci areas within each section were measured directly using a HIPAD digitizing tablet (Houston Instruments, Austin, TX) optically coupled to the microscope. Foci were identified as those areas containing nine or more nuclei or measuring more than 0.1 mm^2 in area. The tissue area, number of foci, and the foci area were all directly recorded at the time of measurement by the use of Bioquant IV image analysis software (R&M Biometrics, Nashville, TN). The number of foci per unit area and volume of liver, the percent foci volume (the volume of liver occupied by foci), and the mean focus area and volume were derived by the quantitative stereological equations of Campbell et al. (1982).

Enzyme Studies

The cyanide-insensitive peroxisomal β-oxidation of palmitoyl CoA procedure of Lazarow (1982) was performed on a 1500 × *g* supernatant fraction of a 20% liver homogenate prepared in 0.25 M sucrose. The initial rate of oxidation was expressed as the amount of nicotinamide adenine dinucleotide formed per minute and normalized either to gram of liver or total liver weight.

Statistics

An analysis of variance test was used to compare body weights, liver-to-body weight ratios, and enzyme data. The enzyme data were tested using the Kruskal-Wallis analysis of variance because the data were not normally distributed. These data were analyzed further by the Bonferroni multiple comparison test after transformation (SAS Institute, Inc., 1985). Foci and related parameters were compared by means of the two-factorial Multivariate Analysis of Variance for Repeated Measures Test on the rank-transformed data because the data were not normally distributed (SAS Institute, Inc., 1985). Groups A and M (the positive control groups) and Groups H and T (the age-matched negative control groups) were combined for analyses of foci data only. The histopathology data were

analyzed by the use of Yates' Corrected Chi-Square (Zar, 1974). For all comparisons an α-level of $p<0.05$ inferred a significant difference between groups. To control for overall experimental error, the alpha level (0.05) was divided by the number of desired comparisons. The computed probability for an individual comparison was compared against the above value, and if the individual comparison probability was less than this value, the comparison was determined to be significant.

SECTION 4

RESULTS

Body and Liver Weight

There were no significant differences in mean terminal body weight between treatment groups after three months. However, there was a significant increase in both the mean liver weight and liver-to-body weight ratio of animals in Group M when compared with those of Groups N through S (Table 3). There were no biologically significant differences in the mean terminal body weight between treatment groups after nine months. However, the mean liver-to-body weight ratio of animals in Group M was significantly greater than that of the animals from Groups N through S.

TABLE 3. TERMINAL BODY WEIGHT, LIVER WEIGHT, AND LIVER-TO-BODY WEIGHT RATIO[a] OF MALE SPRAGUE-DAWLEY RATS FOLLOWING PROMOTION WITH EITHER PHENOBARBITAL OR CTFE TRIMER ACID

	Terminal Body Weight (g)		Terminal Liver Weight (g)		Liver:Body Ratio (%)	
Group	3[b]	9[c]	3[b]	9[c]	3[b]	9[c]
A	506.0 ± 59.1	695.9 ± 39.4	29.2 ± 3.0	34.4 ± 2.3	5.8 ± 0.2	4.9 ± 0.5
B	511.4 ± 37.8	643.1 ± 27.8	24.0 ± 1.7	29.2 ± 1.5	4.7 ± 0.1	4.6 ± 0.4
C	491.3 ± 32.7	753.1 ± 59.2	22.7 ± 4.0	33.3 ± 1.8	4.6 ± 0.5	4.5 ± 0.1
D	546.1 ± 23.0	651.4 ± 26.3	26.3 ± 1.6	32.3 ± 1.8	4.8 ± 0.2	5.0 ± 0.4
E	467.1 ± 11.7	745.8 ± 23.7	22.4 ± 0.8	30.8 ± 1.2	4.8 ± 0.2	4.1 ± 0.2
F	570.4 ± 24.6	648.4 ± 35.2	30.8 ± 3.0	30.9 ± 1.0	5.4 ± 0.4	4.8 ± 0.2
G		686.1 ± 46.4		32.6 ± 3.9		4.7 ± 0.3
H		681.1 ± 14.7		30.0 ± 3.1		4.4 ± 0.5
M	595.3 ± 11.2	691.0 ± 25.2	31.5 ± 2.0	33.8 ± 2.9	5.3 ± 0.4	4.9 ± 0.3
N	473.1 ± 27.1	777.0 ± 19.9	16.5 ± 0.7[d]	25.4 ± 2.3	3.5 ± 0.1[d]	3.3 ± 0.2[d]
O	472.0 ± 22.3	724.8 ± 15.2	18.3 ± 0.8[d]	24.6 ± 0.5[d]	3.7 ± 0.1[d]	3.4 ± 0.1[d]
P	538.0 ± 18.0	661.9 ± 29.5[e]	19.4 ± 0.6[d]	23.0 ± 1.6[d]	3.6 ± 0.02[d]	3.5 ± 0.1[d]
Q	572.8 ± 4.5	801.9 ± 13.1	20.5 ± 0.7[d]	29.1 ± 1.0	3.6 ± 0.1[d]	3.6 ± 0.2[d]
R	486.7 ± 46.7	650.0 ± 44.2	18.2 ± 2.2[d]	25.1 ± 2.5	3.7 ± 0.2[d]	3.8 ± 0.2[d]
S	538.3 ± 42.6	771.7 ± 40.2	19.8 ± 1.9[d]	28.7 ± 0.9	3.7 ± 0.1[d]	3.7 ± 0.2[d]
T		693.7 ± 57.4		27.0 ± 1.3		3.9 ± 0.2

[a] Liver weight/body weight x 100.
[b] Mean ± SEM, N = 3 for all groups except groups E,F,O,P, and R where N = 4.
[c] Mean ± SEM, N = 3 for all groups except groups B,C,D,E,F,G,O,P,Q, and R where N = 4.
[d] Significantly different than Group M at $p<0.05$ by the Kruskal-Wallis analysis of variance test.
[e] Significantly different than Group R at $p<0.05$ by the Kruskal-Wallis analysis of variance test.

Enzyme Data

There were no significant differences in the mean activity of palmitoyl CoA β-oxidation between treatment groups when the initial rate was normalized to a gram of liver. However, when these same data were normalized to total liver weight, differences were noted but were due to increase in liver weight of animals as a function of receiving PB.

Histopathology

The descriptive diagnosis and statistically annotated incidence of histopathologic lesions observed in HE-stained sections of livers from rats in each treatment group is presented in Table 4.

TABLE 4. SUMMARY OF MICROSCOPIC LESIONS INCIDENCE IN LIVER OF SPRAGUE-DAWLEY RATS[a] FOLLOWING INITIATION/PROMOTION FOR NINE MONTHS AND STAINED WITH HEMATOXYLIN AND EOSIN

	Experimental Groups[b]															
Lesion	A	B	C	D	E	F	G	H	M	N	O	P	Q	R	S	T
	3	4	4	4	4	4	4	3	3	3	4	4	4	4	3	3
Clear cell focus	1	0	0	1	0	0	1	0	2	3	3	2	0	0	0	0
Eosinophilic focus	0	0	0	0	0	0	0	0	0	1	2	3	1	0	0	0
Basophilic focus	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Atrophic hepatic cords	3	0[c]	0[c]	0[c]	0[c]	0[c]	0[c]	0[c]	3	0[e]	0[e]	0[e]	0[e]	0[e]	0[e]	0[e]
Steatosis	3	2	4	4	4	4	4	3	2	1	0	2	2	1	2	1
Kuppfer cell pigment	3	4	4	4	4	4	4	3	3	3	4	4	4	4	4	3
Hepatic inflammation	3	2	2	1[c]	3[d,f]	3[d,f]	0[c]	0[c]	3	3	4	4	3	2	1[g,h]	1[g,h]
Bile duct proliferation	2	1	2	1	1	1	1	0	2	3	3	1[i]	1[i]	1[i]	1[i]	0[g,i]
Hepatocytomegaly	0	4[c,d]	4[c,d]	4[c,d]	4[c,d]	4[c,d]	4[c,d]	0	0	0	0	0	0	0	0	0
Neoplastic nodule	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Hepatocytic necrosis	1	0	2	0	0	0	0	0	1	2	0	0	0	0	0	0

[a] Each data cell contains the number of animals affected per treatment group.
[b] Treatments: Refer to Tables 1 and 2 for explanation of treatment groups.
[c] Significantly different from A at $p \leq 0.05$.
[d] Significantly different from H at $p \leq 0.05$.
[e] Significantly different from M at $p \leq 0.05$.
[f] Significantly different from G at $p \leq 0.05$.
[g] Significantly different from O at $p \leq 0.05$.
[h] Significantly different from P at $p \leq 0.05$.
[i] Significantly different from N at $p \leq 0.05$.

Clear cell foci were characterized by randomly dispersed islets of ballooned hepatocytes that were devoid of cytoplasm or that contained pale pink amorphous cytoplasm (Figure 1A). Eosinophilic foci were randomly dispersed in hepatic lobules and contained enlarged hepatocytes with dispersed clumps of eosinophilic cytoplasm (Figure 1B). Collectively, the incidence of clear cell and eosinophilic foci tended to be higher in Groups N, O, and P than in any combination of the other treatment groups. A single basophilic (hyperplastic) focus was seen in the liver of a rat in Group M (positive control, Figure 1C) and contained aggregated hepatocytes that were much smaller than hepatocytes observed in the age-matched controls. Further, these cells contained prominent basophilic cytoplasm within small cytoplasmic compartments. Atrophic hepatic cords were characterized by compressed peripherolobular hepatic cords with small hepatocytes as compared to the larger midzonal and centrolobular hepatocytes.

Figure 2A is a representative section of normal liver. A number of pathological changes were observed in sections from most animal groups. Steatosis or hepatocytic fatty change occurred in liver sections from rats in most treatment groups and appeared to begin in cells as fine microvacuolation. This progressed to coalesced larger smoothly contoured vacuoles, then to ballooned clear or pale pink amorphous cytoplasm (Figure 2B). Nuclei in the hepatocytes with fatty change tended to be eccentric to peripheral in the cells. Kuppfer cells in liver sections from all treatment groups contained prominent gold-to-brown pigment, presumably a hematogenous pigment present from the iron administration (Figure 2C). Hepatic inflammation tended to be observed as periportal infiltrates of lymphocytes, plasma cells, and macrophages with occasional mast cells, neutrophils, and eosinophils. The inflammatory changes tended to be minimally to mildly severe (Figure 3A).

Hepatocytomegaly, particularly centrilobular hepatocytomegaly, was observed as a widely distributed multifocal change in the liver of rats that received CTFE trimer acid and PB, or PB only (Figure 3B). A single well-demarcated carcinomatous neoplastic nodule was observed in a liver section from a hepatectomized rat that received DEN and an intermediate dose of trimer acid (Group O; Figure 3C). The nodule caused peripheral compression of hepatocytes and hepatocytes within the nodule were smaller than other hepatocytes in the section. The nodule hepatocytes were also arranged in disoriented cords. A few animals in four different treatment groups developed scattered foci of necrosis that involved isolated cells or small focal aggregates of hepatocytes.

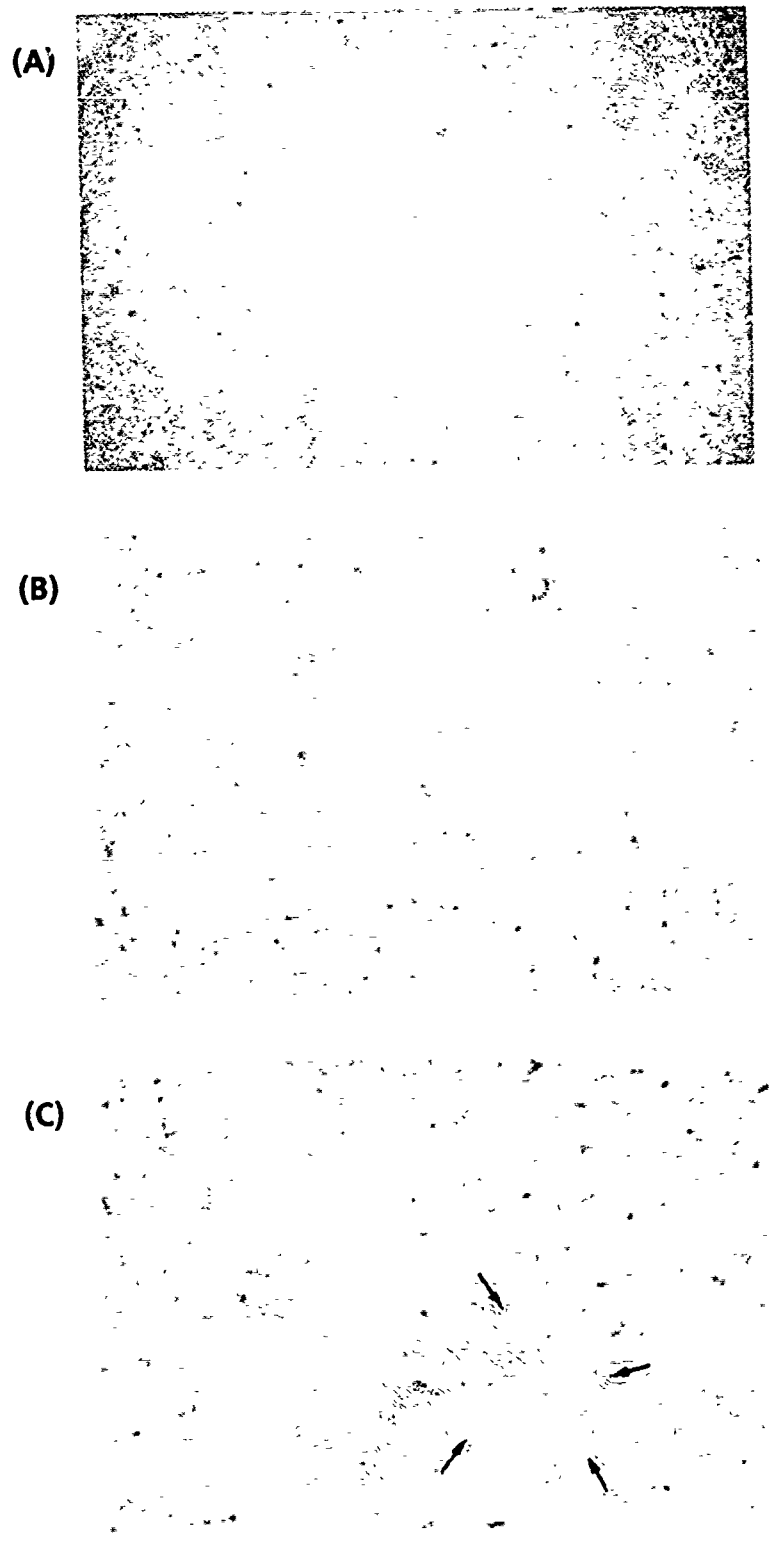


Figure 1. Photomicrographs of Liver Sections Demonstrating the Appearance of Foci Detectable by HE Staining. (A) Clear cell focus with hepatocytic degeneration (25×), (B) Eosinophilic focus (50×), (C) Hyperplastic focus (25×).

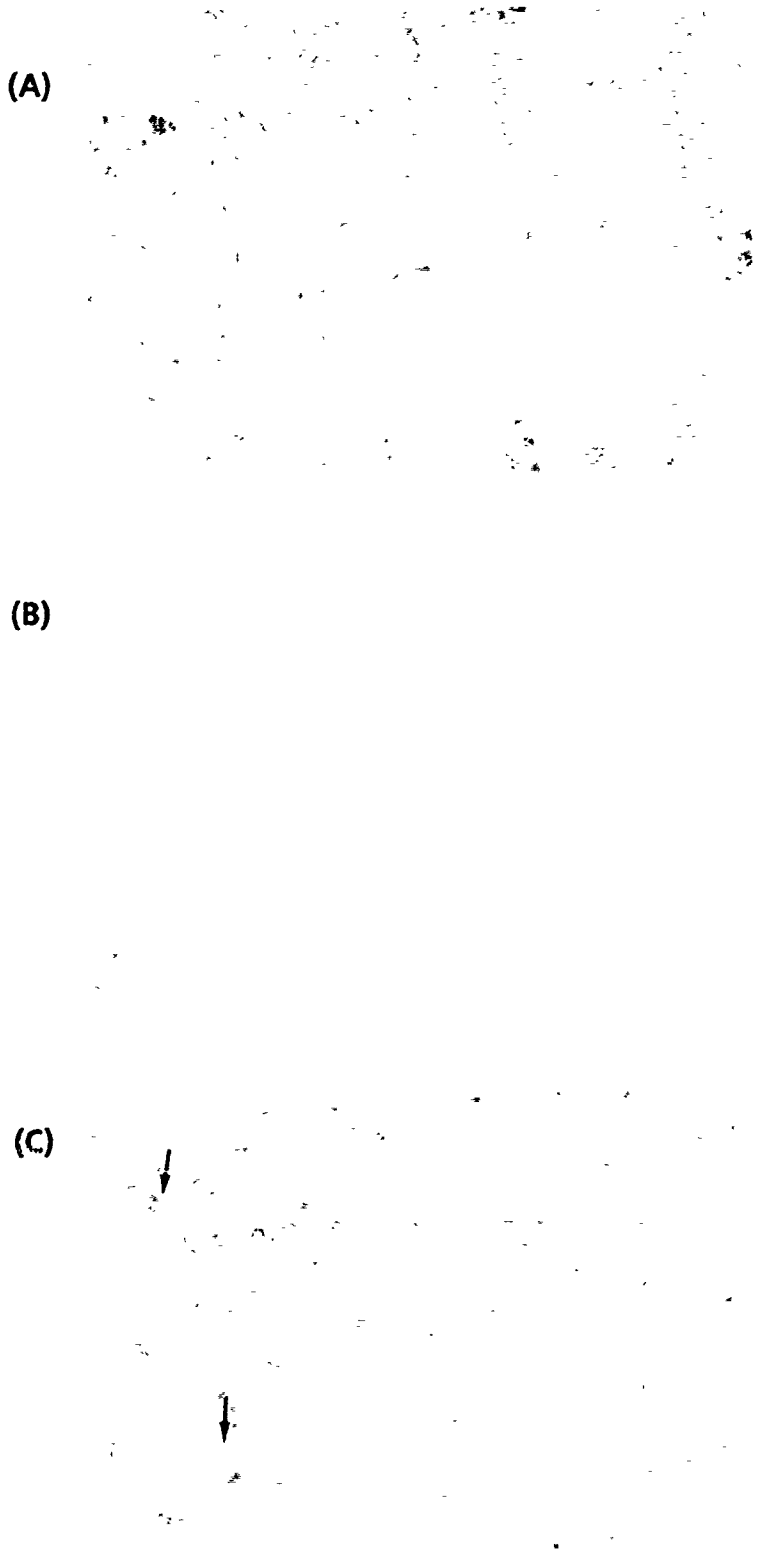


Figure 2. Photomicrographs of Liver Sections Stained with HE Showing Representative Histopathological Lesions. (A) Essentially normal area (100×), (B) Steatosis (50×), (C) Kuppfer cell iron deposits (100×).

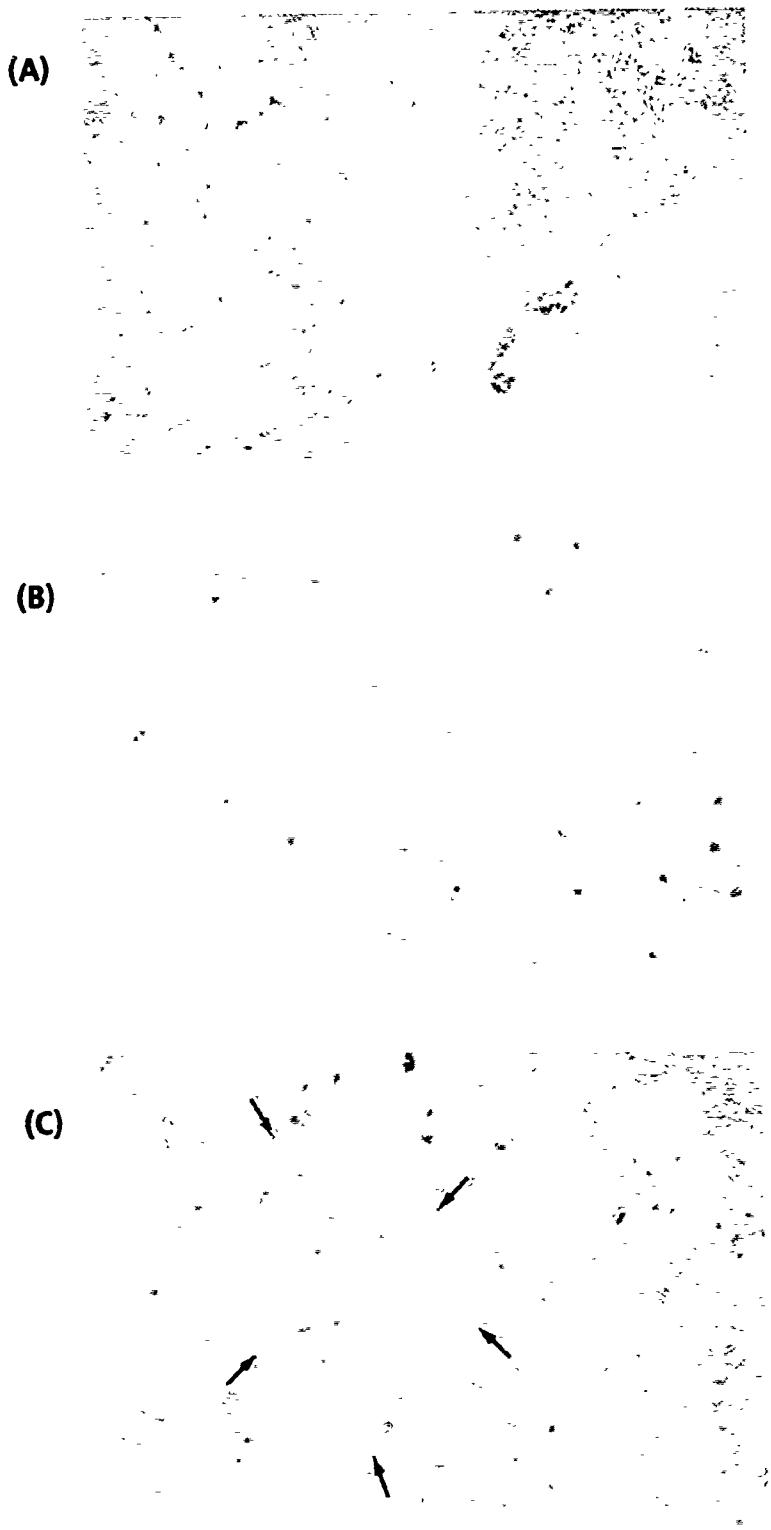


Figure 3. Photomicrographs of Liver Sections Stained with HE Showing Representative Histopathological Lesions. (A) Chronic periportal inflammation (50×), (B) Centrolobular hepatocytomegaly (100×), (C) Neoplastic nodule (50×).

Phenotypic Appearance of Foci

The photomicrographs in Figure 4A-C illustrate the typical appearance of GGTase-positive, ATPase-, and G6Pase-deficient foci, respectively, in sections taken from a single animal from Group N following nine months of promotion with the highest dose of CTFE trimer acid. In comparison, the photomicrographs in Figure 5A-C illustrate the typical appearance of foci expressing these same markers taken from animals from Groups A and M (the positive control groups) following nine months of promotion with PB. No apparent differences in the appearance of these foci among Groups A, M, and N could be detected.

The photomicrographs in Figure 6A-C represent serial sections from the liver of an animal from Group N that was stained for the same three markers as described above. It is clear that this single focus expresses all three markers, and although this was typical of many foci, there were examples of foci that expressed only one or two of the markers.

Quantitation of Altered Foci

After three months of promotion only liver sections stained for the presence of GGTase-positive and iron-deficient foci were examined for the presence of foci (Tables 5 and 6). Liver sections stained with HE from most animals revealed alterations of hepatocyte morphology and staining. These altered hepatocytes, located primarily in centrolobular regions of lobules, were enlarged with increased amounts of eosinophilic-staining cytoplasm filled with numerous variably sized vacuoles. These foci were not quantified because of the atypical morphology and staining of the hepatocytes. However, liver sections from animals that received trimer acid as a promoter (Groups N through Q) appeared normal. Slides stained for the presence of ATPase- and G6Pase-deficient foci could not be interpreted because of weak staining. Slides stained for the presence of glycogen-positive foci by the PAS stain did not contain any detectable foci.

The quantitative stereology of liver sections from animals in treatment groups that received initiation with trimer acid and promotion with PB for three months (Groups B through E) did not reveal any significant increase in any of the parameters when compared to those of controls. However, liver sections from animals receiving promotion with trimer acid for three months (Groups N through P) and stained for GGTase-positive foci revealed elevations in foci per square and cubic centimeter in Groups N and O above those of the control Groups Q and R (Table 5). No elevations in these parameters were noted in liver sections stained for the presence of iron-deficient foci, but a significant difference in the percent foci volume was noted for Groups N and O when compared with those of control Groups R and S, respectively (Table 6).

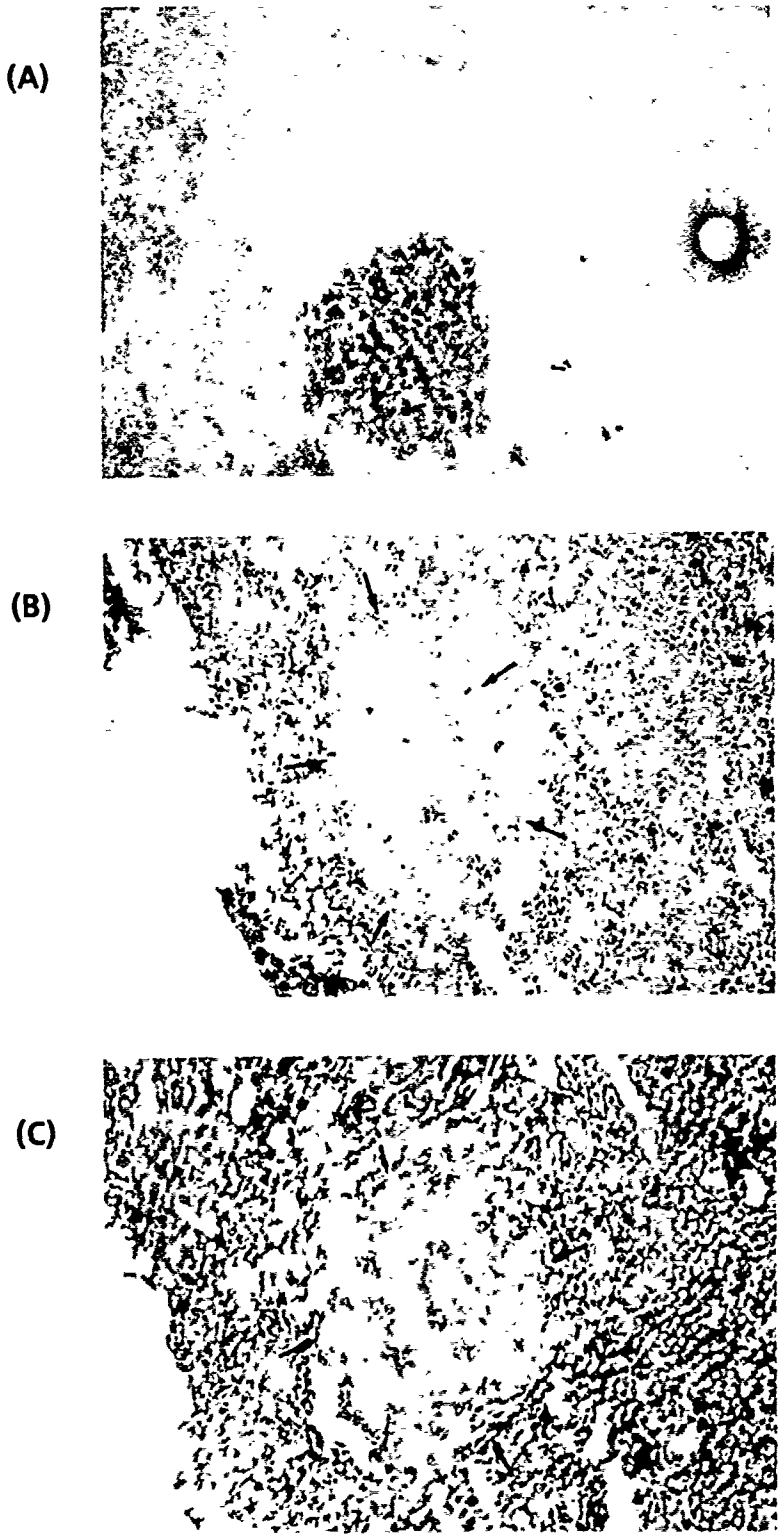


Figure 4. Photomicrographs of Liver Sections Taken from Animals in Group N Demonstrating the Phenotypic Appearance of Foci. (A) GGTase-positive focus, (B) ATPase-deficient focus, (C) G6Pase-deficient focus (25 ×).

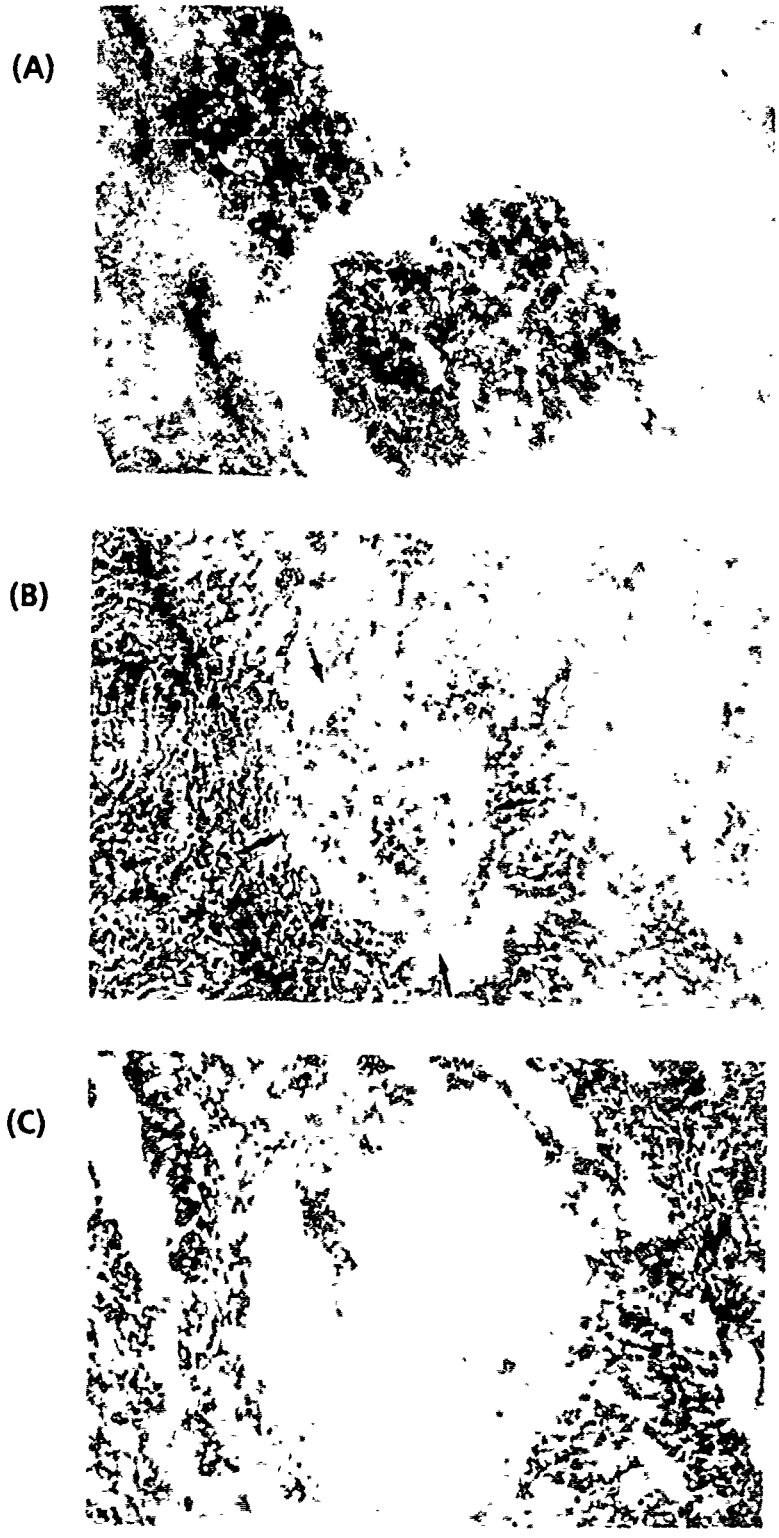


Figure 5. Photomicrographs of Liver Sections Taken from Animals in Groups A and M Demonstrating the Phenotypic Appearance of Foci. (A) GGTase-positive focus, (B) ATPase-deficient focus, (C) G6Pase-deficient focus (25 ×).

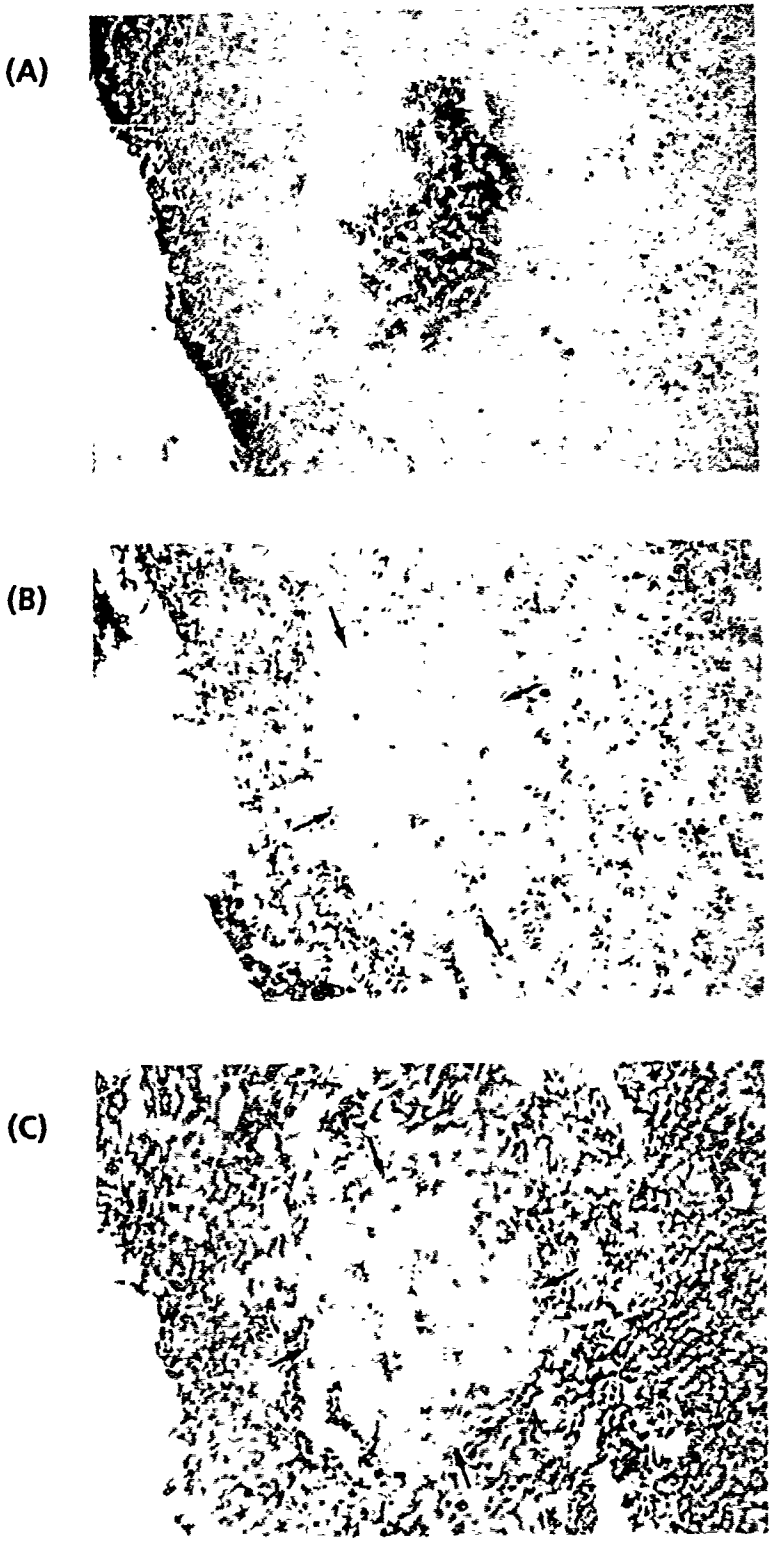


Figure 6. Photomicrographs of Liver Sections Taken from an Animal in Group N Showing Simultaneous Expression of Three Different Markers in a Single Focus. (A) GGTase-positive focus, (B) ATPase-deficient focus, (C) G6Pase-deficient focus (25 x).

TABLE 5. PARAMETERS[a] OF GGTase-POSITIVE FOCI DETECTED IN THE LIVERS OF ANIMALS FOLLOWING THREE MONTHS OF PROMOTION

Group	Foci/cm^2	Foci/cm^3	% Foci Volume	Mean Area (mm^2)	Mean Volume (mm^3)
AM	7.6 ± 0.5	258.8 ± 22.1	0.25 ± 0.03	0.033 ± 0.002	0.010 ± 0.001
B	0.5 ± 0.5	14.3 ± 14.3	0.03 ± 0.03	0.018 ± 0.018	0.006 ± 0.006
C	0.4 ± 0.4	11.9 ± 11.9	0.01 ± 0.01	0.011 ± 0.011	0.004 ± 0.004
D	0.2 ± 0.2	5.5 ± 5.5	0.004 ± 0.004	0.009 ± 0.009	0.003 ± 0.003
E	0.3 ± 0.2	22.2 ± 13.0	0.003 ± 0.002	0.004 ± 0.002	0.001 ± 0.0004
F	n.d.[b]	n.d.	n.d.	n.d.	n.d.
N	1.9 ± 0.4[c,d]	82.7 ± 9.4[c,d]	0.04 ± 0.01	0.018 ± 0.003	0.004 ± 0.001
O	2.0 ± 0.3[c,d]	98.7 ± 14.1[c,d,e]	0.03 ± 0.01	0.014 ± 0.001	0.006 ± 0.001
P	0.7 ± 0.4	34.5 ± 20.6	0.01 ± 0.01	0.007 ± 0.004	0.001 ± 0.001
Q	n.d.	n.d.	n.d.	n.d.	n.d.
R	n.d.	n.d.	n.d.	n.d.	n.d.
S	1.0 ± 0.3	43.9 ± 9.7	0.02 ± 0.01	0.018 ± 0.004	0.004 ± 0.001

[a] Values represent the mean of three animals ± 1 SEM except for groups E,F,O,P, and R where N = 4.
[b] n.d. = no foci detected
[c] Significantly different from Q at $p<0.05$ by Two-factorial Analysis of Variance for Repeated Measures test.
[d] Significantly different from R at $p<0.05$ by Two-factorial Analysis of Variance for Repeated Measures test.
[e] Significantly different from S at $p<0.05$ by Two-factorial Analysis of Variance for Repeated Measures test.

TABLE 6. PARAMETERS[a] OF IRON-DEFICIENT FOCI DETECTED IN THE LIVERS OF ANIMALS FOLLOWING THREE MONTHS OF PROMOTION.

Group	Foci/cm^2	Foci/cm^3	% Foci Volume	Mean Area (mm^2)	Mean Volume (mm^3)
AM	7.4 ± 1.1	154.7 ± 31.1	0.64 ± 0.07	0.091 ± 0.010	0.045 ± 0.008
B	2.7 ± 0.1	56.7 ± 3.8	0.21 ± 0.02	0.078 ± 0.008	0.038 ± 0.008
C	3.0 ± 0.3	62.8 ± 6.1	0.23 ± 0.03	0.078 ± 0.005	0.037 ± 0.006
D	2.6 ± 0.3	52.9 ± 6.2	0.22 ± 0.03	0.085 ± 0.006	0.042 ± 0.004
E	2.2 ± 0.3	46.8 ± 7.0	0.18 ± 0.02	0.084 ± 0.009	0.041 ± 0.007
F	0.4 ± 0.2	10.4 ± 3.9	0.03 ± 0.01	0.045 ± 0.016	0.020 ± 0.009
N	3.4 ± 0.5	70.5 ± 13.7	0.28 ± 0.07[b]	0.084 ± 0.013	0.043 ± 0.010
O	2.5 ± 0.5	53.3 ± 10.4	0.19 ± 0.04[c]	0.076 ± 0.006	0.036 ± 0.005
P	2.8 ± 0.5	61.5 ± 9.0	0.23 ± 0.06	0.076 ± 0.009	0.035 ± 0.005
Q	1.6 ± 0.5	32.6 ± 7.5	0.13 ± 0.07	0.075 ± 0.015	0.037 ± 0.011
R	1.8 ± 0.7	40.5 ± 18.5	0.13 ± 0.04	0.059 ± 0.023	0.029 ± 0.013
S	4.3 ± 0.4	85.2 ± 11.0	0.43 ± 0.01	0.101 ± 0.007	0.053 ± 0.006

[a] Values represent the mean from three animals ± 1 SEM, except for groups E,F,O,P, and R where N = 4.
[b] Significantly different from R at $p<0.05$ by Two-factorial Analysis of Variance for Repeated Measures Test.
[c] Significantly different from S at $p<0.05$ by Two-factorial Analysis of Variance for Repeated Measures Test.

Quantitation of glycogen-positive foci was not accomplished at nine months because no foci were detectable. However, all liver sections that were stained for the presence of all other markers and with HE were examined and the results are presented in Tables 7 through 11.

TABLE 7. PARAMETERS[a] OF GGTase-POSITIVE FOCI DETECTED IN THE LIVERS OF ANIMALS FOLLOWING NINE MONTHS OF PROMOTION

Group	Foci/cm^2	Foci/cm^3	% Foci Volume	Mean Area (mm^2)	Mean Volume (mm^3)
AM	10.7 ± 2.0	297.8 ± 58.9	0.69 ± 0.23	0.060 ± 0.010	0.024 ± 0.006
B	n.d.[b]	n.d.	n.d.	n.d.	n.d.
C	0.5 ± 0.3	15.1 ± 8.7	0.02 ± 0.0	0.019 ± 0.011	0.006 ± 0.003
D	1.4 ± 0.8	51.6 ± 30.9	0.10 ± 0.07	0.056 ± 0.043	0.019 ± 0.016
E	0.6 ± 0.2	15.9 ± 6.9	0.02 ± 0.01	0.035 ± 0.015	0.014 ± 0.007
F	0.3 ± 0.3	9.9 ± 9.9	0.01 ± 0.01	0.006 ± 0.006	0.002 ± 0.002
G	0.6 ± 0.3	16.9 ± 9.8	0.02 ± 0.02	0.020 ± 0.012	0.007 ± 0.004
N	5.2 ± 1.3[c,d,e]	189.8 ± 51.4[c,d,e]	0.18 ± 0.09[c,d,e]	0.033 ± 0.010	0.010 ± 0.004
O	3.3 ± 1.3[c,d,e]	123.0 ± 45.3[c,d,e]	0.09 ± 0.05	0.024 ± 0.004	0.007 ± 0.001
P	1.5 ± 0.7	43.8 ± 22.0	0.09 ± 0.06	0.037 ± 0.018	0.014 ± 0.007
Q	0.2 ± 0.2	6.1 ± 6.1	0.01 ± 0.01	0.009 ± 0.009	0.003 ± 0.003
R	0.1 ± 0.1	5.0 ± 5.0	0.002 ± 0.002	0.005 ± 0.005	0.001 ± 0.001
S	2.2 ± 0.6	70.9 ± 13.7	0.09 ± 0.03	0.037 ± 0.005	0.012 ± 0.003
HT	0.3 ± 0.3	8.6 ± 8.6	0.02 ± 0.02	0.009 ± 0.009	0.003 ± 0.003

[a] Values represent the mean of four animals ± 1 SEM except for groups A,H,M,N, and T where N = 3.
[b] n.d. = no foci detected
[c] Significantly different from HT at $p<0.05$ by Two-factorial Analysis of Variance for Repeated Measures Test.
[d] Significantly different from Q at $p<0.05$ by Two-factorial Analysis of Variance for Repeated Measures Test.
[e] Significantly different from R at $p<0.05$ by Two-factorial Analysis of Variance for Repeated Measures Test.

TABLE 8. PARAMETERS[a] OF ATPase-DEFICIENT FOCI DETECTED IN THE LIVERS OF ANIMALS FOLLOWING NINE MONTHS OF PROMOTION

Group	Foci/cm^2	Foci/cm^3	% Foci Volume	Mean Area (mm^2)	Mean Volume (mm^3)
AM	5.5 ± 1.4	122.2 ± 33.1	0.44 ± 0.11	0.080 ± 0.001	0.037 ± 0.001
B	0.7 ± 0.3	17.1 ± 7.1	0.05 ± 0.02	0.058 ± 0.027	0.030 ± 0.018
C	1.1 ± 0.1	23.8 ± 3.8	0.09 ± 0.01	0.083 ± 0.011	0.042 ± 0.009
D	1.0 ± 0.4	16.7 ± 7.4	0.14 ± 0.06	0.114 ± 0.059	0.088 ± 0.059
E	1.3 ± 0.3	26.5 ± 6.7	0.11 ± 0.02	0.087 ± 0.009	0.045 ± 0.007
F	1.1 ± 0.4	18.7 ± 7.1	0.11 ± 0.05	0.078 ± 0.029	0.039 ± 0.013
G	1.4 ± 0.5	27.5 ± 10.5	0.14 ± 0.05	0.073 ± 0.024	0.039 ± 0.013
N	7.3 ± 1.0[b,c,d,e]	164.9 ± 18.5[b,c,d,e]	0.51 ± 0.10[b,c,d,e]	0.070 ± 0.004	0.030 ± 0.003
O	2.6 ± 0.8[b]	56.8 ± 19.4[b,d]	0.23 ± 0.07[b]	0.089 ± 0.007	0.044 ± 0.006[b]
P	1.7 ± 0.8	31.4 ± 15.3	0.17 ± 0.09	0.069 ± 0.023	0.026 ± 0.013
Q	0.9 ± 0.4	20.3 ± 8.7	0.08 ± 0.04	0.072 ± 0.033	0.036 ± 0.019
R	0.6 ± 0.3	13.4 ± 7.1	0.05 ± 0.02	0.070 ± 0.030	0.040 ± 0.019
S	1.3 ± 0.7	30.4 ± 13.2	0.11 ± 0.04	0.080 ± 0.006	0.040 ± 0.004
HT	0.5 ± 0.4	12.6 ± 9.7	0.03 ± 0.02	0.025 ± 0.018	0.013 ± 0.010

[a] Values represent the mean of four animals ± 1 SEM except for groups A,H,M,N, and T where N = 3.
[b] Significantly different from HT at $p<0.05$ by Two-factorial Analysis of Variance for Repeated Measures Test.
[c] Significantly different from Q at $p<0.05$ by Two-factorial Analysis of Variance for Repeated Measures Test.
[d] Significantly different from R at $p<0.05$ by Two-factorial Analysis of Variance for Repeated Measures Test.
[e] Significantly different from S at $p<0.05$ by Two-factorial Analysis of Variance for Repeated Measures Test.

TABLE 9. PARAMETERS[a] OF G6Pase-DEFICIENT FOCI DETECTED IN THE LIVERS OF ANIMALS FOLLOWING NINE MONTHS OF PROMOTION

Group	Foci/cm^2	Foci/cm^3	% Foci Volume	Mean Area (mm^2)	Mean Volume (mm^3)
AM	7.6 ± 1.0	153.8 ± 20.8	0.74 ± 0.11	0.097 ± 0.006	0.048 ± 0.004
B	0.7 ± 0.2	10.6 ± 4.2	0.09 ± 0.03	0.104 ± 0.037[c]	0.068 ± 0.026[c]
C	1.3 ± 0.7	31.5 ± 19.7	0.13 ± 0.08	0.090 ± 0.047	0.047 ± 0.026
D	1.1 ± 0.5	19.2 ± 8.5	0.18 ± 0.11	0.104 ± 0.046	0.061 ± 0.030[c]
E	0.6 ± 0.3	10.3 ± 4.9	0.07 ± 0.03	0.093 ± 0.032[b,c]	0.059 ± 0.020[c]
F	1.6 ± 0.7	36.7 ± 15.4	0.11 ± 0.05	0.048 ± 0.018	0.021 ± 0.009
G	0.9 ± 0.2	17.1 ± 4.2	0.10 ± 0.03	0.108 ± 0.014	0.064 ± 0.012
N	10.2 ± 0.2[c,d,e,f]	243.0 ± 9.7[c,d,e,f]	0.73 ± 0.07[c,d,e,f]	0.072 ± 0.006	0.030 ± 0.004
O	5.1 ± 1.3[c,d,e]	118.2 ± 32.8[c,e]	0.38 ± 0.08[c,e]	0.080 ± 0.012	0.038 ± 0.009
P	3.8 ± 0.8[c,e]	81.8 ± 15.2[c,e]	0.34 ± 0.10[c,e]	0.084 ± 0.008	0.039 ± 0.005
Q	1.6 ± 0.4	41.1 ± 8.3	0.14 ± 0.06	0.079 ± 0.025	0.038 ± 0.014
R	0.6 ± 0.6	14.0 ± 14.0	0.04 ± 0.04	0.018 ± 0.018	0.008 ± 0.008
S	2.2 ± 0.5	42.2 ± 10.9	0.24 ± 0.04	0.116 ± 0.016	0.068 ± 0.016
HT	0.2 ± 0.2	4.3 ± 4.3	0.02 ± 0.02	0.018 ± 0.018	0.008 ± 0.008

[a] Values represent the mean of four animals ± 1 SEM except for groups A,H,M,N, and T where N = 3.
[b] Significantly different from F at $p<0.05$ by Two-factorial Analysis of Variance for Repeated Measures Test.
[c] Significantly different from HT at $p<0.05$ by Two-factorial Analysis of Variance for Repeated Measures Test.
[d] Significantly different from Q at $p<0.05$ by Two-factorial Analysis of Variance for Repeated Measures Test.
[e] Significantly different from R at $p<0.05$ by Two-factorial Analysis of Variance for Repeated Measures Test.
[f] Significantly different from S at $p<0.05$ by Two-factorial Analysis of Variance for Repeated Measures Test.

TABLE 10. PARAMETERS[a] OF HEMATOXYLIN- AND EOSIN-DETECTABLE FOCI IN THE LIVERS OF ANIMALS FOLLOWING NINE MONTHS OF PROMOTION

Group	Foci/cm^2	Foci/cm^3	% Foci Volume	Mean Area (mm^2)	Mean Volume (mm^3)
AM	5.5 ± 1.4	95.1 ± 23.7	0.89 ± 0.24	0.157 ± 0.009	0.091 ± 0.006
B	n.d.[b]	n.d.	n.d.	n.d.	n.d.
C	n.d.	n.d.	n.d.	n.d.	n.d.
D	n.d.	n.d.	n.d.	n.d.	n.d.
E	n.d.	n.d.	n.d.	n.d.	n.d.
F	n.d.	n.d.	n.d.	n.d.	n.d.
G	n.d.	n.d.	n.d.	n.d.	n.d.
N	8.7 ± 1.0[c,d,e]	172.1 ± 23.8[c,d,e]	1.03 ± 0.13[c,d,e,f]	0.120 ± 0.009[c]	0.061 ± 0.007[c]
O	5.5 ± 0.5[c,d,e]	112.6 ± 11.6[c,d]	0.61 ± 0.09[c,d,e]	0.111 ± 0.010[c]	0.054 ± 0.005[c]
P	8.1 ± 1.4[c,d,e]	167.0 ± 35.2[c,d,e]	0.89 ± 0.10[c,d,e,f]	0.118 ± 0.016[c]	0.060 ± 0.011[c]
Q	3.5 ± 0.6	79.5 ± 12.9	0.31 ± 0.08	0.087 ± 0.012	0.039 ± 0.007
R	0.3 ± 0.2	7.1 ± 4.7	0.03 ± 0.01	0.077 ± 0.030	0.044 ± 0.019
S	1.3 ± 0.6	28.3 ± 11.6	0.11 ± 0.06	0.063 ± 0.021	0.028 ± 0.010
HT	n.d.	n.d.	n.d.	n.d.	n.d.

[a] Values represent the mean of four animals ± 1 SEM except for groups A,H,M,N, and T where N = 3.
[b] n.d. = no foci detected
[c] Significantly different from HT at $p<0.05$ by Two-factorial Analysis of Variance for Repeated Measures Test.
[d] Significantly different from R at $p<0.05$ by Two-factorial Analysis of Variance for Repeated Measures Test.
[e] Significantly different from S at $p<0.05$ by Two-factorial Analysis of Variance for Repeated Measures Test.
[f] Significantly different from Q at $p<0.05$ by Two-factorial Analysis of Variance for Repeated Measures Test.

TABLE 11. PARAMETERS[a] OF IRON-DEFICIENT FOCI DETECTED IN THE LIVERS OF ANIMALS FOLLOWING NINE MONTHS OF PROMOTION

Group	Foci/cm^2	Foci/cm^3	% Foci Volume	Mean Area (mm^2)	Mean Volume (mm^3)
AM	8.0 ± 0.6	147.8 ± 11.0	0.92 ± 0.12	0.115 ± 0.010	0.063 ± 0.006
B	1.9 ± 0.2[b]	36.0 ± 4.9[b]	0.18 ± 0.03[b]	0.097 ± 0.008	0.052 ± 0.007
C	0.7 ± 0.3	13.3 ± 6.1	0.07 ± 0.03	0.078 ± 0.026	0.042 ± 0.015
D	0.5 ± 0.3	11.0 ± 5.5	0.05 ± 0.02	0.069 ± 0.024	0.034 ± 0.012
E	1.0 ± 0.4	18.0 ± 8.7	0.10 ± 0.04	0.102 ± 0.005	0.057 ± 0.005[b]
F	0.4 ± 0.4	7.6 ± 7.6	0.04 ± 0.04	0.026 ± 0.026	0.014 ± 0.014
G	0.8 ± 0.3	15.4 ± 6.0	0.07 ± 0.02	0.094 ± 0.012	0.052 ± 0.010
N	4.9 ± 0.8[c]	88.9 ± 14.9[c]	0.58 ± 0.09[c,d]	0.119 ± 0.010[c]	0.066 ± 0.007[c]
O	4.4 ± 0.7[c]	88.3 ± 14.5[c]	0.45 ± 0.06[c]	0.104 ± 0.008	0.052 ± 0.004
P	2.4 ± 0.6	42.1 ± 9.8	0.30 ± 0.07[c]	0.123 ± 0.009[c]	0.070 ± 0.006[c]
Q	3.3 ± 0.4	64.8 ± 8.8	0.37 ± 0.07	0.111 ± 0.019	0.059 ± 0.013
R	1.8 ± 0.4	33.6 ± 7.1	0.20 ± 0.04	0.112 ± 0.009	0.064 ± 0.008
S	2.6 ± 0.6	46.8 ± 9.7	0.30 ± 0.08	0.113 ± 0.010	0.063 ± 0.008
HT	0.8 ± 0.4	16.1 ± 7.9	0.06 ± 0.03	0.057 ± 0.020	0.030 ± 0.012

[a] Values represent the mean of four animals ± 1 SEM except for groups A,H,M,N, and T where N = 3.
[b] Significantly different from F at $p<0.05$ by Two-factorial Analysis of Variance for Repeated Measures Test.
[c] Significantly different from HT at $p<0.05$ by Two-factorial Analysis of Variance for Repeated Measures Test.
[d] Significantly different from R at $p<0.05$ by Two-factorial Analysis of Variance for Repeated Measures Test.

Liver sections from animals initiated with trimer acid and promoted with PB for nine months (Groups B through E) did not reveal a significant increase in foci per square or cubic centimeter. Staining for the presence of G6Pase- and iron-deficient foci in some groups showed a significant increase in mean area and volume over those of some control groups but were probably not biologically significant.

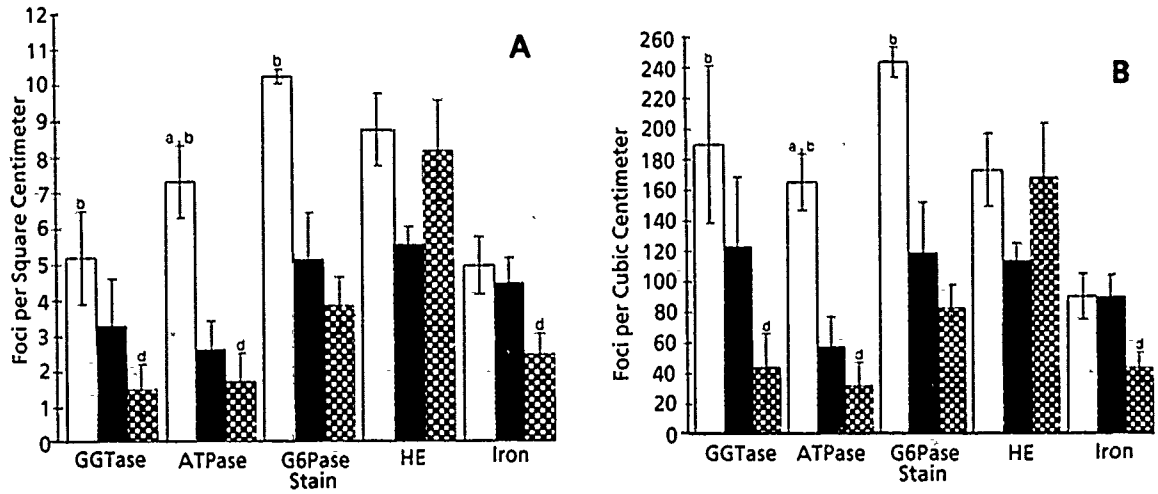
Liver sections from animals promoted with three different dosage levels of trimer acid for nine months following initiation with DEN (Groups N through P) revealed statistically significant increases in many of the parameters when compared with those of the control groups (Q through S and HT). The differences in Groups N through P over the control groups varied with the staining procedure used to detect foci. For example, the parameters of iron-deficient foci were for the most part significantly greater only from those in the age-matched negative control (Group HT), whereas the parameters of ATPase- and G6Pase-deficient foci were significantly greater from those in all control groups (Q through S and HT).

Liver sections from animals that received the highest dose of trimer acid as promoter (Group N) revealed a significant increase in foci per square and cubic centimeter and percent foci volume when compared to that of the different control groups (Q through S, HT). Staining with HE, and for iron-deficient foci, showed significant increases in mean foci area and volume over those of the age-matched negative control animals (Group HT) only.

Most of the measured parameters of foci from livers of animals that received the intermediate dose of trimer acid (Group O) were significantly greater than those of the control groups. The measurements of foci from liver sections of animals receiving the lowest dose of trimer acid (Group P) and stained for GGTase-positive foci and ATPase-deficient foci were not significantly different from those of control animals. However, staining of liver sections of Group P animals for the presence of G6Pase-deficient foci, and with HE, revealed significant differences in most of the measurements over those of the various control groups. The percent foci volume, mean area, and mean volume of iron-deficient foci from Group P animals were increased signficantly over those of Group HT only.

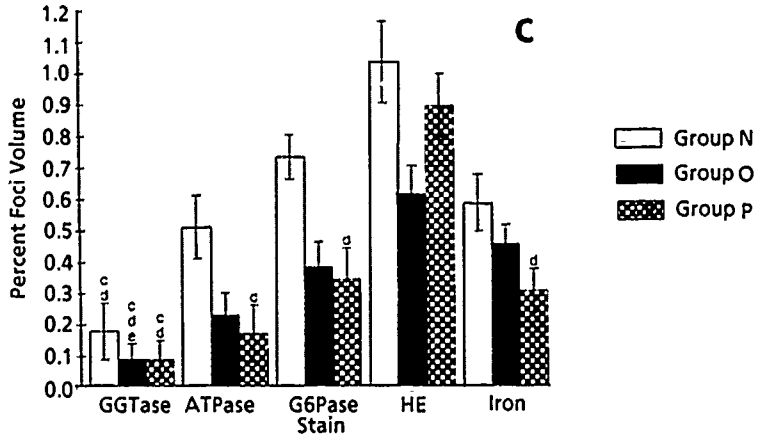
A comparison of the five measurements of foci parameters from Groups N through P (Figure 7) illustrates the differences between these three treatment groups. Although a dose response was apparent in many cases, significant differences between the three treatment groups were not always present. In the case of foci per square centimeter (Figure 7A) an apparent dose response was evident with each staining technique except for HE. A dose response was also evident in the case of foci per cubic centimeter in those liver sections stained with the three histochemical methods (Figure 7B). Sections stained for ATPase-, G6Pase- and iron-deficient foci revealed a dose response in percent foci

volume (Figure 7C). No apparent dose response was noted for the mean area or volume of the foci detected by any of the staining techniques (Figure 7D-E).



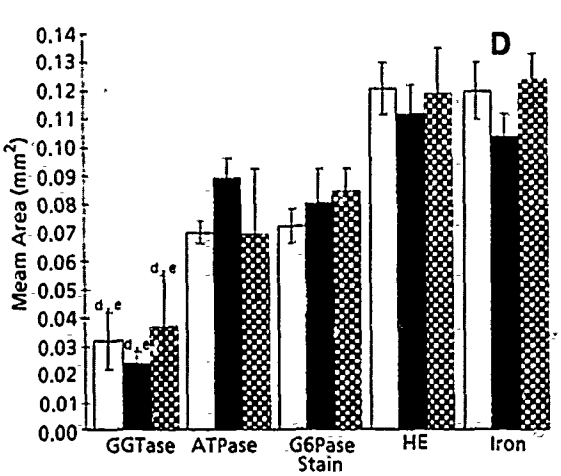
a = Significantly greater than group O for this stain ($p<0.05$)
b = Significantly greater than group P for this stain ($p<0.05$)
d = Significantly less than those detected in this group with HE stain ($p<0.05$)

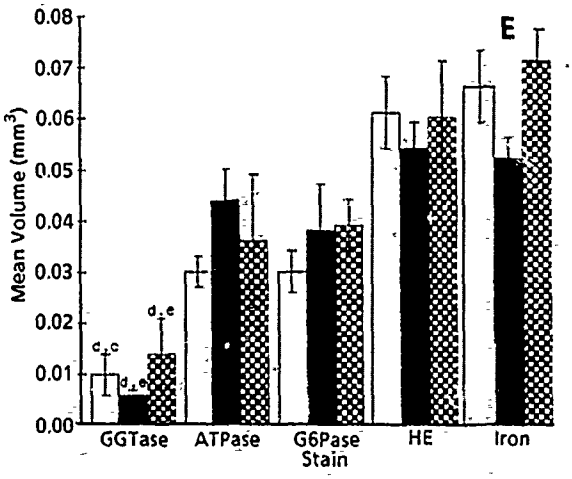
a = Significantly greater than group O for this stain ($p<0.05$)
b = Significantly greater than group P for this stain ($p<0.05$)
d = Significantly less than those detected in this group with HE stain ($p<0.05$)



c = Significantly less than that of the same group stained for G6Pase ($p<0.05$)
d = Significantly less than that of the same group stained with HE ($p<0.05$)
e = Significantly less than than that of the same group stained for iron ($p<0.05$)

Figure 7A-E. Comparison of Computed Parameters of Foci from Livers of Animals in Groups Receiving Promotion for Nine Months with CTFE Trimer Acid and Stained for Five Markers.





d = Significantly less than that detected in this group with HE stain ($p<0.05$)
e = Significantly less than that detected in this group with iron stain ($p<0.05$)

d = Significantly less than that detected in this group with HE stain ($p<0.05$)
e = Significantly less than that detected in this group with iron stain ($p<0.05$)

Figure 7A-E. Continued.

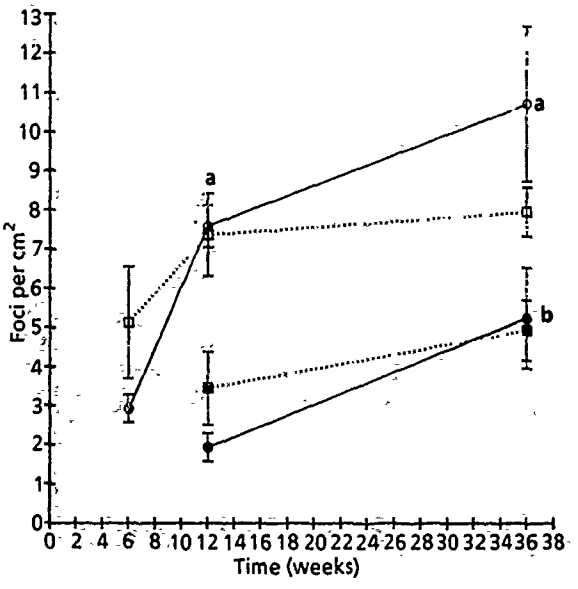
The mean area and volume of the GGTase-positive foci from animals in Groups N through P were smaller than those of foci detected in liver sections stained with HE or iron-deficient foci (Figure 7E). The percent of the liver occupied by GGTase-positive foci (percent foci volume) was also lower than the percent of the liver occupied by foci detectable with the other markers except for those showing ATPase-deficiency (Figure 7C). Hematoxylin and eosin staining of liver sections in animals receiving the lowest promotion dose of trimer acid (Group P) revealed significant increases in foci per square and cubic centimeter and percent foci volume when compared to those that were detectable by the other staining methods (Figure 7A-C, respectively).

Growth of Foci

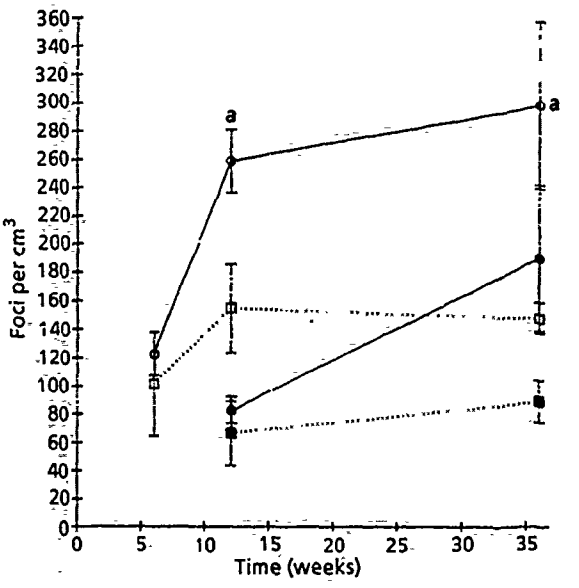
Figure 8 compares the measurements of GGTase-positive and iron-deficient foci parameters from livers of animals in both the positive control group (Group AM) and the group receiving the highest dose of CTFE trimer acid as the promoter (Group N) at the 12- and 36-week time points in the present study. Data have been included for the six-week time period for comparison purposes only and were taken from Godin and Wall (1990).

Measurements of GGTase-positive foci increased in both groups of animals over time, but not all of the increases were statistically significant. All measurements except those of mean area and volume of the GGTase-positive foci were significantly smaller for animals in Group N than for animals in Group AM at 12 weeks (three months), but by week 36 (nine months) only the percent foci volume of Group N was significantly less than that of Group AM. Although the number of GGTase-positive foci per square centimeter appears to increase at approximately the same rate in both groups, the

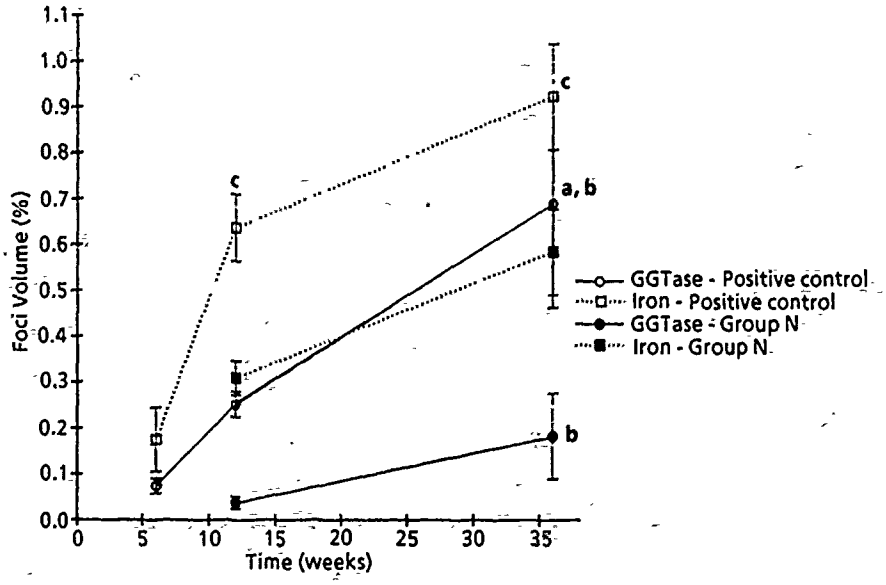
increase in GGTase-positive foci per cubic centimeter appeared to increase at a more rapid rate in livers of Group N animals than for those in Group AM. The percent foci volume and the mean area and volume of GGTase-positive foci in Group N did not increase as fast as those for Group AM. The increase of percent foci volume and mean area and volume was nearly linear with respect to time in Group AM animals.



a = GGTase significantly different from week 6 ($p<0.05$).
b = GGTase significantly different from week 12 ($p<0.05$).

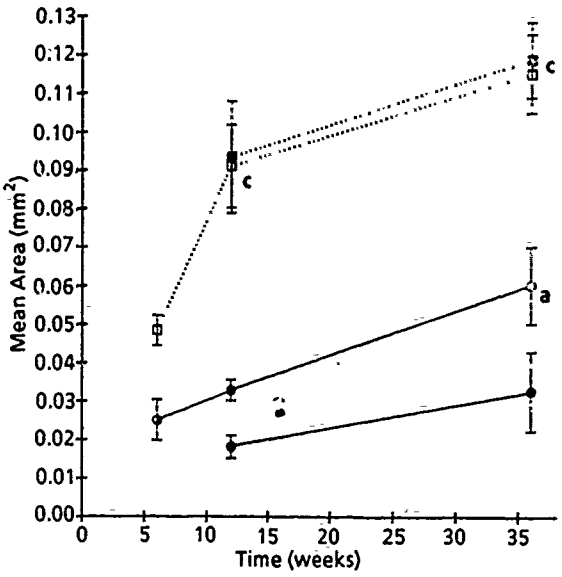


a = GGTase significantly different from week 6 ($p<0.05$).

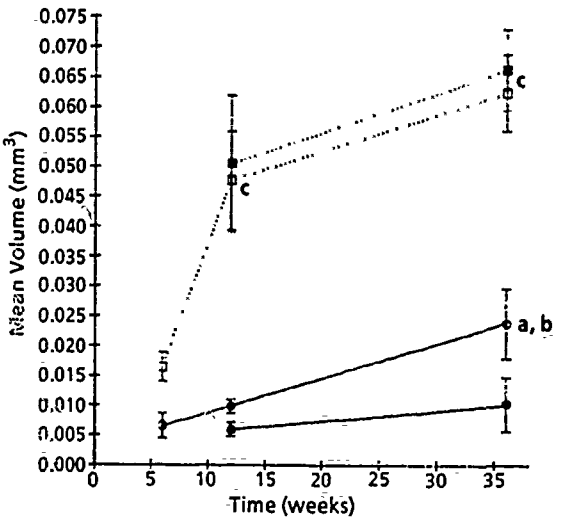


a = GGTase significantly different from week 6 ($p<0.05$).
b = GGTase significantly different from week 12 ($p<0.05$).
c = Iron significantly different from week 6 ($p<0.05$).

Figure 8. Change in Parameters with Time of GGTase-Positive and Iron-Deficient Foci from Livers of Animals Receiving Promotion with Either PB or CTFE Trimer Acid.



a = GGTase significantly different from week 6 ($p<0.05$).
c = Iron significantly different from week 6 ($p<0.05$).



a = GGTase significantly different from week 6 ($p<0.05$).
b = GGTase significantly different from week 12 ($p<0.05$).
c = Iron significantly different from week 6 ($p<0.05$).

Figure 8. Continued.

There was a trend for all measured parameters except the foci per cubic centimeter to increase with respect to time in the case of iron-deficient foci. Although all of the measurements of foci parameters in Group N tended to be lower than those of Group AM at each time point, only the percent foci volume of animals in Group N was signficantly smaller than that of animals in Group AM, and only at 12 weeks. The mean foci area and mean foci volume for both groups of animals at the 12- and 36-week time periods are nearly identical. It is interesting to note that the rate of increase for the percent foci volume, mean area, and mean volume, is nearly identical for Group N and Group AM.

SECTION 5

DISCUSSION

The administration of CTFE oligomer (a mixture of C6 and C8 CTFE oligomers) has resulted in peroxisomal proliferation when administered by different routes (Kinkead et al., 1990; DelRaso, unpublished data). Peroxisomal proliferators cause an inhibition of mitochondrial fatty acid oxidation in rat liver (Bone et al., 1982; Horie and Suga, 1985; Elcombe and Mitchell, 1986; Draye and Vamecq, 1987; Foxworthy and Eacho, 1988; Eacho and Foxworthy, 1988) and, therefore, greatly increase the number of hepatic peroxisomes and the amount of peroxisomal enzymes involved in fatty acid oxidation (Sharma et al., 1988). It has been suggested that the mechanism of mitochondrial inhibition involves the formation of a metabolically inert CoA ester derivative from peroxisome proliferators.

The administration of peroxisome proliferators such as hypolipidemic agents and phthalate esters has been shown to be hepatocarcinogenic in rodents and has been substantiated by numerous studies (National Toxicology Program, 1976; Reddy and Rao, 1977; Reddy and Qureshi, 1979; Reddy et al., 1979; Reddy et al., 1980; Reddy et al., 1982; Rao et al., 1984). Studies by Reddy et al. (1986) and Tomaszewski et al. (1986) have concluded that peroxisome proliferation is correlated with the formation of hepatic tumors when the degree of peroxisome proliferation in their respective studies was compared to tumor incidence in historical bioassay data. However, these studies used doses and routes of dosing that were different from those used in the original bioassays. Marsman et al. (1988) duplicated conditions of the original bioassay for both Wy-14,643 and di(2-ethylhexyl)phthalate and concluded that the degree of peroxisome proliferation was poorly correlated with hepatocarcinogenicity, but that the degree of replicative DNA synthesis was strongly correlated with tumor development.

Although the mechanism by which peroxisome proliferators cause hepatocarcinogenesis is unknown, it is clear that these chemicals must be chronically administered to cause tumor formation (Stott, 1988). Furthermore, there have been no examples, to our knowledge, of the induction of either preneoplastic foci or tumors without the concurrent demonstration of a several-fold elevation in peroxisomal β-oxidation rate and increased relative liver weight following such chronic administration.

In the present study the chronic administration of CTFE trimer acid did not cause an increase in either peroxisomal β-oxidation rate or the relative liver weight at either the three- or nine-month time point. A slight increase in the rate of peroxisomal β-oxidation, but not relative liver weight, over that of control was noted in a previous study in which CTFE trimer acid was chronically administered

to male F-344 rats for three months by oral gavage (Kinkead et al., 1990), no difference in the rate between treated and control was noted after an additional three months of dosing. The lack of induction in the present study may reflect the difference in the routes of administration or of the strain of rat used.

On the basis of the above findings CTFE trimer acid, a weak peroxisome proliferator, would not be expected to cause the development of either preneoplastic foci or tumors. When tested for its ability to initiate or promote hepatocarcinogenesis, there was no increase in any of the measured parameters in livers of animals initiated with CTFE trimer acid and promoted with PB. Because of these observations CTFE trimer acid is probably not genotoxic. This lack of genotoxicity is not surprising in light of studies examining the genotoxic potential of CTFE trimer acid that have clearly shown that this compound does not induce mutagenic changes (Godin et al., unpublished data).

When examined for its ability to promote DEN-initiated hepatocytes, a significant increase in the number of foci per unit area and volume occurred in the livers of animals after three months of promotion with CTFE trimer acid. These values, as well as the percent foci volume, the mean area, and the mean volume, tended to increase during the subsequent 24 weeks of treatment. Significant increases in foci/cm^2, foci/cm^3, and percent foci volume above those of control groups using five out of the six staining techniques were clearly evident after an additional 24 weeks of promotion; staining for glycogen-positive foci did not demonstrate detectable foci. Of particular interest was the observation of GGTase-positive foci in all animals receiving CTFE trimer acid as a tumor promoter. Tumors induced by other peroxisome proliferators do not express this marker (Rao et al., 1982; 1987). To our knowledge, this represents the first report of an increase in the number of these GGTase-positive foci following the administration of a peroxisome proliferator. The higher incidence of clear cell and eosinophilic foci in groups of hepatectomized rats that were initiated with DEN and promoted with various concentrations of trimer acid as well as the changes in foci quantitative stereology suggest that under the conditions of this study CTFE may have promoting activity.

The induction of foci in this study is interesting because no significant increase in the rate of peroxisomal oxidation of palmitoyl CoA, when this rate was expressed in terms of micromoles per minute per gram, was observed in any CTFE trimer acid-promoted animals at the two sampling time points in the study. It is possible however, that in the present study an early rise in peroxisomal oxidation occurred prior to the three-month sampling point. Because there was no increase in liver weight of CTFE trimer acid-promoted animals (evidence for replicative DNA synthesis) and no increase in the rate of hepatic peroxisomal β-oxidation, the finding of increased GGTase-positive foci in animals treated with CTFE trimer acid as a tumor promoter may indicate that a different mechanism for tumor promotion unrelated to the events of peroxisome proliferation and/or replicative DNA synthesis may exist for this class of chemicals.

SECTION 6

ACKNOWLEDGMENTS

We thank Ms. Trish Deiser for assistance with the partial hepatectomies and Ms. Gloria Neely and Ms. Sharon Wagner for preparation of frozen sections.

SECTION 7

REFERENCES

Bedi, K.S. and R.W. Horobin. 1976. An alcohol-soluble Schiff's reagent: A histochemical application of the complex between Schiff's reagent and phosphotungstic acid. *Histochemistry* 48:153-159.

Bone, A.J., S.A. Sherratt, D.M. Turnbull, and H. Osmundsen. 1982. Increased activity of peroxisomal β-oxidation in rat liver caused by ethyl-2{5(4-chlorophenyl)pentyl}-oxiran-2-carboxylate: An inhibitor of β-oxidation. *Biochem. Biophys. Res. Commun.* 104:708-712.

Campbell, H.A., H.C. Pitot, V.R. Potter, and B.A. Laishes. 1982. Application of quantitative stereology to the evaluation of enzyme-altered foci in rat liver. *Cancer Res.* 42:465-472.

Draye, J.P. and J. Vamecq. 1987. The inhibition by valproic acid of the mitochondrial oxidation of monocarboxylic and omega-hydroxymonocarboxylic acids: Possible implications for the metabolism of gamma-aminobutyric acid. *J. Biochem.* 102:235-242.

Eacho, P.I. and P.S. Foxworthy 1988. Inhibition of hepatic fatty acid oxidation by benzafibrate and benzafibroyl CoA. *Biochem. Biophys. Res. Commun.* 157:1148-1153.

Elcombe, C.R. 1985. Species differences in carcinogenicity and peroxisome proliferation due to trichloroethylene: A biochemical human hazard assessment. *Arch. Toxicol.* 8(Suppl.):6-17.

Elcombe, C.R. and A.M. Mitchell. 1986. Peroxisome proliferation due to di(2-ethylhexyl)phthalate (DEHP): Species differences and possible mechanisms. Environ. Health Perspect.70:211-219.

Foxworthy, P.S. and P.I. Eacho. 1988. Inhibition of hepatic fatty acid oxidation as carnitine palmitoyltransferase I by the peroxisome proliferator 2-hydroxy-2-propyl-[6-(tetrazol-5-yl)hydroxy]acetophenone. *Biochem. J.* 252:409-414.

Godin, C.S. and H.G. Wall. 1990. Development of an initiation/promotion assay to detect enzyme altered hepatocytes. AAMRL-SR-90-501. Harry G. Armstrong Medical Research Laboratory, Wright-Patterson Air Force Base, OH.

Gupta, R.C., S.K. Goel, K. Early, B. Singh, and J.K. Reddy. 1985. ^{32}P-postlabeling analysis of peroxisome proliferator-DNA adduct formation in rat liver *in vivo* and hepatocytes *in vitro*. *Carcinogenesis* 6:933-936.

Harrison, E.H., J.S. Lane, S. Luking, M.J. Van Rafelghem, and M.E. Andersen. 1988. Perfluoro-*n*-decanoic acid: Induction of peroxisomal β-oxidation by a fatty acid with dioxin-like toxicity. *Lipids* 23:115-119.

Hartig, F., H.G. Stegmeier, M. Ozel, and H.D. Fahimi. 1982. Study of liver enzymes: Peroxisome proliferation and tumor rates in rats at the end of carcinogenicity studies with benzafibrate and clofibrate. *Ann. N.Y. Acad. Sci.* 386:464-467.

Higgins, G.M. and R.M. Anderson. 1931. Experimental pathology of the rat liver. *Arch. Pathol.* 12:186-202.

Hirota, N. and G.M. Williams. 1979. The sensitivity and heterogeneity of histochemical markers for altered foci involved in liver carcinogenesis. *Am. J. Pathol.* 95:317-324.

Horie, S. and T. Suga. 1985. Enhancement of peroxisomal β-oxidation in the liver of rats and mice treated with valproic acid. *Biochem. Pharmacol.* 34:1357-1362.

Ishii, H., S. Fukumori, S. Horie, and T. Suga. 1980. Effects of fat content in the diet on hepatic peroxisomes of the rat. *Biochim. Biophys. Acta* 617:1-11.

Kawashima, Y., H. Katoh, S. Nakajima, H. Kozuka, and M. Uchiyama. 1984. Effects of 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid on peroxisomal enzymes in rat liver. *Biochem. Pharmacol.* 33:241-245.

Kinkead, E.R., S.K. Bunger, and R.W. Wolfe. 1990. Repeated-dose gavage study of chlorotrifluoroethylene acids. In: R.S. Kutzman, H.G. Wall, and A.Vinegar, eds. *1989 Toxic Hazards Research Unit Annual Report.* AAMRL-TR-90-051, Wright-Patterson Air Force Base, OH: Armstrong Aerospace Medical Research Laboratory; NMRI-90-92, Bethesda, MD: Naval Medical Research Institute.

Kinkead, E.R., E.C. Kimmel, H.G. Wall, R.B. Conolly, R.S. Kutzman, R. Whitmire, and D.R. Mattie. 1990. Subchronic studies of chlorotrifluoroethylene. *Inh. Toxicol.* 2:431-449.

Lalwani, N.D., M.K. Reddy, S.A. Qureshi, C.R. Sartori, Y. Abiko, and J.K. Reddy. 1983. Evaluation of selected hypolipidemic agents for the induction of peroxisomal enzymes and peroxisome proliferation in the rat liver. *Human Toxicol.* 2:27-48.

Lazarow, P.B. 1982. Assay of peroxisomal β-oxidation of fatty acids. *Methods Enzymol.* 72:315-321.

Marsman, D.S., R.C. Cattley, J.G. Conway, and J.A. Popp. 1988. Relationship of hepatic peroxisome proliferation and replicative DNA synthesis to the hepatocarcinogenicity of the peroxisome proliferators di(2-ethylhexyl)phthalate and [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid (Wy-14,643) in rats. *Cancer Res.* 48:6739-6744.

McCarthy, R.D. 1964. Mammalian metabolism of straight-chain saturated hydrocarbons. *Biochim. Biophys. Acta* 84:74-79.

Mochizuki, Y., K. Furukawa, and N. Sawada. 1982. Effects of various concentrations of ethyl-p-chlorophenoxyisobutyrate (clofibrate) on diethylnitrosamine-induced hepatic tumorigenesis in the rat. *Carcinogenesis* 3:1027-1029.

Moody, D.E. and J.K. Reddy. 1978. Hepatic peroxisome (microbody) proliferation in rats fed plasticisers and related compounds. *Toxicol. Appl. Pharmacol.* 45:497-504.

National Toxicology Program (NTP). 1976. Carcinogenesis Bioassay of Trichloroethylene. DHEW Publ. No. (NIH) 76-802.

National Toxicology Program (NTP). 1982. Carcinogenesis Bioassay of Di(2- ethylhexyl)phthalate in F-344 Rats and B6C3F1 Mice. NIH Publ. No. 82-1773.

Olson, C.T., M.E. Andersen, M.E. George, M.J. Van Rafelghem, and A.M. Back. 1982. The toxicology of perfluorodecanoic acid in rodents. AAMRL-TR-82-101. Proceedings of the Thirteenth Annual Conference on Environmental Toxicology, Air Force Aerospace Medical Research Laboratory Technical Report, p.287-303.

Parnell, M.J., L.D. Koller, J.H. Exon, and J.M. Arnzen. 1986. Trichloroacetic acid effects on rat liver peroxisomes and enzyme-altered foci. *Environ. Health Perspect.* 69:73-79.

Rao, M.S., N.D. Lalwani, D.G. Scarpelli, and J.K. Reddy. 1982. The abscence of gamma-glutamyl transpeptidase activity in putative preneoplastic lesions and in hepatocellular carcinomas induced in rats by the hypolipidemic peroxisomal proliferator Wy 14,643. *Carcinogenesis.* 3:1231-1233.

Rao, M.S., N.D. Lalwani, T.K. Watanabe, and J.K. Reddy. 1984. Inhibitory effect of antioxidant ethoxyquin and 2(3)-tert-butyl-4-hydroxyanisole on hepatic tumorigenesis in rats fed ciprofibrate, a peroxisome proliferator. *Cancer Res.* 44:1072-1076.

Rao, M.S., N.Usuda, V. Subbarao, and J.K. Reddy. 1987. Absence of gamma- glutamyl transpeptidase in neoplastic lesions induced in the liver of male F-344 rats by DEHP, a peroxisome proliferator. *Carcinogenesis* 8:1347-1351.

Reddy, J.K., D.L. Azarnoff, and C.E. Hignite. 1980. Hypolipidemic hepatic peroxisome proliferators form a novel class of chemical carcinogens. *Nature* (London) 283:397-398.

Reddy, J.K., N.D. Lalwani, M.K. Reddy, and S.A. Qureshi. 1982. Excessive accumulation of autofluorescent lipofuscin in the liver during hepatocarcinogenesis by methyl clofenopate and other hypolipidemic peroxisome proliferators. *Cancer Res.* 42:259-266.

Reddy, J.K., D.E. Moody, D.L. Azarnoff, and M.S. Rao. 1976. Di-(2- ethylhexyl)phthalate: An industrial plasticizer induces hypolipidemia and enhances hepatic catalase and carnitine acetyltransferase activities in rats and mice. *Life Sci.* 18:941-946.

Reddy, J.K. and S.A. Qureshi. 1979. Tumorigenicity of the hypolipidemic peroxisome proliferator ethyl-p-chlorophenoxyiso-butyrate (clofibrate) in rats. *Brit. J. Cancer* 40:476-482.

Reddy, J.K. and M.S. Rao. 1977. Malignant tumors in rats fed nafenopin, a hepatic peroxisome proliferator. *J. Natl. Cancer Inst.* 59:1645-1650.

Reddy, J.K. and M.S. Rao. 1978. Enhancement by Wy-14,643, a hepatic peroxisome proliferator of diethylnitrosamine-initiated hepatic tumorigenesis in the rat. *Brit. J. Cancer* 38:537-543.

Reddy, J.K., M.S. Rao, D.L. Azarnoff, and S. Sell. 1979. Mitogenic and carcinogenic effects of a hypolipidemic peroxisome proliferator, (4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio)acetic acid (Wy-14,643), in rat and mouse liver. *Cancer Res.* 39:152-161.

Reddy, J.K., M.K. Reddy, M.I. Usman, N.D. Lalwani, and M.S. Rao. 1986. Comparison of hepatic peroxisome proliferative effect and its implications for hepatocarcinogenicity of phthalate esters, di(2-ehtylhexyl)phthalate and di(2-ethylhexyl)adipate with a hypolipidemic drug. *Environ. Health Perspect.* 65:317-327.

Rutenburg, A.M., H. Kim, J.W. Fischbein, J.S. Hanker, H.L. Wasserkrug, and A.M. Seligman. 1969. Histochemical and ultrastructural demonstration of gamma-glutamyltranspeptidase activity. *J. Histochem. Cytochem.* 17:517-526.

SAS Institute, Inc. 1985. SAS User's Guide:Statistics, Version 5 Edition. Cary, NC: SAS Institute, Inc.

Schulte-Hermann, R., G. Ohde, J. Schuppler, and I. Timmermann-Trosiener. 1981. Enhanced proliferation of putative preneoplastic cells in rat liver following treatment with the tumor promoters

phenobarbital, hexachlorocyclohexane, steroid compounds and nafenopin. *Cancer Res.* 41:2556-2562.

Sharma, R., B.G. Lake, J. Foster, and G.G. Gibson. 1988. Microsomal cytochrome P-452 induction and peroxisome proliferation by hypolipidaemic agents in rat liver: A mechanistic inter-relationship. *Biochem. Pharmacol.* 37:1193-1201.

Stott, W.T. 1988. Chemically induced proliferation of peroxisomes: Implications for risk assessment. *Reg. Toxicol. and Pharmacol.* 8:125-159.

Tomaszewski, K.E., D.K. Agarwal, and R.L. Melnick. 1986. *In vitro* steady state levels of hydrogen peroxide after exposure of male F344 rats and female B6C3F1 mice to hepatic peroxisome proliferators. *Carcinogenesis* 7:1871-1877.

Vainio, H., M. Linnainmaa, J. Kahonen, E. Nickels, E. Hietanen, J. Marniemi, and P. Peltonen. 1983. Hypolipidemia and peroxisome proliferation induced by phenoxyacetic acid herbicides in rats. *Biochem. Pharmacol.* 32:2775-2779.

Van Rafelghem, M.J. 1985. The Toxicity and Hepatic Ultrastructure Effects of Perfluoro-*n*-decanoic Acid in Four Rodent Species. Dissertation. Fairborn, OH: Wright State University.

Wachstein, M. and E. Meisel. 1957. Histochemistry of hepatic phosphatases at physiologic pH. *Am. J. Clin. Pathol.* 27:13-23.

Wachstein, M. and E. Meisel. 1958. On the histochemical demonstration of glucose-6-phosphatase. *J. Histochem.* 4:753.

Warren, J.R., V.F. Simmon, and J.K. Reddy. 1980. Properties of hypolipidemic peroxisome proliferators in the lymphocyte [^{3}H]thymidine and *Salmonella* mutagenesis assays. *Cancer Res.* 40:36-41.

Zar, J.H. 1974. *Biostatistical Analysis*, Chapter 9, pp105-106. Englewood Cliffs, NJ: Prentice Hall.

☆U.S. Government Printing Office: 1991 — 648-069/40222

SECTION 8

QUALITY ASSURANCE

The study, "The Evaluation of the Initiation/Promotion Potential of CTFE Trimer Acid," was conducted by the NSI Technology Services Corporation, Toxic Hazards Research Unit under the guidance of the Environmental Protection Agency's Good Laboratory Practices Guidelines, 40CFR PART 792. No claim will be made that this was a "GLP" study as no attempt was made to adhere to the strict requirements of these guidelines. The various phases of this study were inspected by members of the Quality Assurance Unit. Results of these inspections were reported directly to the Study Director at the close of each inspection.

DATE OF INSPECTION:	ITEM INSPECTED:
Animal Group A	
March 28, 1989	Phenobarbitol dosing
June 6, 1989	Iron dosing
June 21, 1989	12 week sacrifice, frozen sections, enzyme assay
November 21, 1989	Iron dosing
December 6, 1989	36 week sacrifice
Animal Group N	
April 4, 1989	CTFE IP dosing
June 13, 1989	Iron dosing
June 27, 1989	12 week sacrifice, frozen sections, enzyme assay
November 28, 1989	Iron dosing
December 12, 1989	36 week sacrifice, frozen sections, enzyme assay
Animal Groups A, N	
August 15, 1989	Liver section staining
August 22, 1989	Liver section staining
August 29, 1989	Liver section staining
October 3, 1989	Liver section staining
October 23, 1989	Liver section staining
August 22-29, 1990	Final report review

The Quality Assurance Unit has determined by review process that this report accurately describes those methods and standard operating procedures required by the protocol and that the reported results accurately reflect the raw data obtained during the course of the study. No discrepancies were found that would alter the interpretation presented in this Final Report.

M. G. Schneider
QA Coordinator
Toxic Hazards Research Unit
Date 29 Aug 90